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GLUTAMATE TRANSPORTER ASSOCIATED PROTEINS AND METHODS OF USE THEREOF

RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e)(1) to U.S. Provisional Application Serial No. 60/161,007, filed October 23, 1999, and to U.S. Provisional Application Serial No. 60/206,157, filed on May 22, 2000, each herein incorporated by reference in their entirety.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

This invention was made with Government support under NS33958 and NS70151, awarded, by the National Institutes of Health (NINDS). The Government may have certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates generally to protein-protein interactions and more specifically to Glutamate Transporter Associated Proteins involved in mediating glutamate transport, chloride transport and cytoskeletal stability and their association with glutamate transporter proteins.

BACKGROUND OF THE INVENTION

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system, acting on postsynaptic ionotropic glutamate receptors (particularly NMDA and AMPA receptors). In addition, glutamate stimulates a subset of metabotropic glutamate receptors (particularly the group I metabotropic glutamate receptors mGluR1a and mGluR5) concentrated in the postsynaptic membrane. The timely removal of glutamate from the synaptic cleft is critical to preventing desensitization resulting from continued exposure of the postsynaptic receptors to glutamate. Removal of glutamate from the synaptic cleft is mediated by a class of molecules known as glutamate transporter proteins located on surrounding astroglia and neurons. Five distinct, high-affinity, sodium-dependent glutamate transporters have been identified in animal and human central nervous system. Rat GLAST,

GLT-1, EAAC1 (EAAT1, EAAT2 and EATT3, respectively, in human), EAAT4 and EAAT5 differ in structure, pharmacological properties and tissue distribution.

Glutamate transport is a sodium- and potassium-coupled process capable of concentrating intracellular glutamate up to 10,000-fold compared with the extracellular environment. The stoichiometry of the process has been studied and at several models exist proposing various ionic exchanges. In one model derived from salamander retinal glial cells, the transport process is coupled to the co-transport of two sodium ions and the counter-transport of one potassium ion and one hydroxyl ion. (Bouvier et al. (1992), Nature 360:471-474). Another model proposes that with EAAC1, one glutamate is co-transported with three sodium ions and one hydrogen ion, with the counter-transport of one potassium ion (Zerangue *et al,* Nautre (1996) 383:634-637). Yet another study suggests that two sodium ions are co-transported with one glutamate molecule (Hart *et al.,* Science (1998) 280:2112-2114).

The cloning of glutamate transporter subtypes and detailed electrophysiological studies of these proteins reveals that glutamate transporters also possess channel-like properties. The conduct chloride flux is not thermodynamically coupled to substrate transport, although at transportable substrate is required for the chloride conductance. The binding of glutamate to the transporter may change its conformational state to form the chloride channel.

In addition to their possible role in development and learning (due to their potential for modulating normal synaptic transmission), the regulation of synaptic glutamate transporters is likely to play an important role in acute and chronic neurological processes. They can be involved through the disruption of synaptic transmission as well as through glutamate mediated excitotoxicity. Several diseases are associated with disruptions in glutamate transport.

Loss of cerebellar Purkinje cell is the hall mark of several inherited neurodegenerative diseases, including the trinucleotide repeat diseases such as spinocerebellar ataxia type 1 (SCA1), and is commonly associated with neurotoxicity of chronic ethanol ingestion, and with certain paraneoplastic neurological disorders. Although the molecular event that initiates the disease is known-- a trinucleotide repeat -- the cellular mechanisms responsible for Purkinje cell degeneration is not

known. The selective loss of glutamate transporters such as EAAT4 could make the protein an attractive candidate for a downstream event.

Similarly, dysregulation of glutamate transporter EAAC1 could also have pathological consequences. EAAC1 has the unusual localization to GABA pre-synaptic terminals. This transport could serve as a precursor transporter, supplying extracellular glutamate for GABA re-synthesis. GABA normally is synthesized, via glutamate amino decarboxylase, from glutamate. The source of this glutamate has been traditionally thought to be cellular glutamate. However, the unique localization of the glutamate transporter to GABA terminals suggests that these transporters supply precurser glutamate for GABA re-synthesis. Thus, EAAC1 could serve as an important step in GABAergic neurotransmission. Modulation of GABAergic metabolism is associated with a number of neurological disorders, including epilepsy, tremors, and spasticity. In addition, some theories of schizophrenia include disturbances of glutamate and GABA metabolism.

Accordingly, there is a need in the art for compounds that regulate glutamate transport and in particular, compounds and molecules that interact with glutamate transporter proteins.

SUMMARY OF THE INVENTION

The present invention provides a family of proteins that interact with glutamate transporter proteins. Through their interaction with glutamate transporter proteins, Glutamate Transporter Associated Proteins modulate glutamate transport, and also effect cytoskeletal organization and stability as well as chloride flux.

In one embodiment of the invention, there is provided a substantially pure polypeptide characterized as modulating intracellular glutamate transport, interacting with a glutamate transporter protein, and having an expression pattern in the brain. In addition, the polypeptide can have at least one PDZ domain, at least one regulatory G-protein domain, at lest one pleckstrin homology domain, at least one proline-rich domain and at least one guanine exchange factor domain. The polypeptide can have at least one pleckstrin homology domain, at least one spectrin repeat and at least one α-actinin domain.

In an additional embodiment of the invention, there is provided a substantially pure polypeptide characterized as modulating intracellular glutamate transport; interacting with a glutamate transporter protein; having an expression pattern in neural non-neuronal tissues; having at least one kinase C domains; having four transmembrane domains; and being hydrophobic.

In another embodiment of the invention, there is provided a substantially pure polypeptide having an amino acid sequence as set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 or conservative variants thereof.

In still another embodiment of the invention, there is provided an isolated polynucleotide selected from the group consisting of: (a) a polynucleotide encoding a polypeptide having an amino acid sequence as set forth in SEQ ID NO:2; (b) a polynucleotide of (a), wherein T can be U; (c) a polynucleotide complementary to (a) or (b); (d) a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:1; (e) degenerate variants of (a), (b), (c) or (d); and (f) a fragment of (a), (b), (c), (d) or (e) having at least 15 base pairs and hybridizes to a polynucleotide encoding a polypeptide as set forth in SEQ ID NO:2.

In yet another embodiment of the invention, there is provided an isolated polynucleotide selected from the group consisting of: (a) a polynucleotide encoding a polypeptide having an amino acid sequence as set forth in SEQ ID NO:4; (b) a polynucleotide of (a), wherein T can be U; (c) a polynucleotide complementary to (a) or (b); (d) a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:3; (e) degenerate variants of (a), (b), (c) or (d); and (e) a fragment of (a), (b), (c), (d) or (e) having at least 15 base pairs and hybridizes to a polynucleotide encoding a polypeptide as set forth in SEQ ID NO:4.

In still another embodiment of the invention, there is provided an isolated polynucleotide selected from the group consisting of: (a) a polynucleotide encoding a polypeptide having an amino acid sequence as set forth in SEQ ID NO:6; (b) a polynucleotide of (a), wherein T can be U; (c) a polynucleotide complementary to (a) or (b); (d) a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:5; (e) degenerate variants of (a), (b), (c) or (d); and (f) a fragment of (a), (b), (c),

(d) or (e) having at least 15 base pairs and hybridizes to a polynucleotide encoding a polypeptide as set forth in SEQ ID NO:6.

In still a further embodiment of the invention, there is provided an antibody that binds to a Glutamate Transporter Associated Protein or binds to immunoreactive fragments thereof. The antibody can be polyclonal or monoclonal.

In yet another embodiment of the invention, there is provided an expression vector comprising a polynucleotide encoding Glutamate Transporter Associated Protein, *e.g.*, SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, or complementary nucleotides thereof and fragments thereof. The vectors can be virus derived or plasmid derived.

In another embodiment of the invention, there is provided a method for producing a Glutamate Transporter Associated Protein polypeptide by culturing a host cell containing a nucleotide encoding a Glutamate Transporter Associated Protein under conditions suitable for the expression of the polypeptide and recovering the polypeptide from the host cell culture.

In another embodiment of the invention, there is provided a substantially pure polypeptide that interacts with the amino acid sequence QEAELTLP (SEQ ID NO:9) or amino acid sequence GRGGNESVM (SEQ ID NO:10).

In still another embodiment of the invention, there is provided a substantially pure polypeptide that interacts with the amino acid sequence set forth in SEQ ID NO:12.

In still another embodiment of the invention, there is provided a substantially pure polypeptide that interacts with the amino acid sequence set forth in SEQ ID NO:13.

In an addition embodiment of the invention, there is provided a method for identifying a compound that modulates a cellular response mediated by a Glutamate Transporter Associated Protein. The method includes incubating the compound with a cell expressing a Glutamate Transporter Associated Protein and a glutamate transporter protein under conditions sufficient to permit the components to interact

and comparing a cellular response in the cell incubated with the compound with the cellular response of a cell not incubated with the compound.

In yet another embodiment of the invention, there is provided a method for identifying a compound that inhibits an interaction between a Glutamate Transporter Associated Protein and a glutamate transporter protein. The method includes contacting a Glutamate Transporter Associated Protein with a glutamate transporter protein in the presence of the compound and comparing the formation of a Glutamate Transporter Associated Protein-glutamate transporter protein complex in the presence of the compound with a formation of the complex in the absence of the compound.

In still another embodiment of the invention, there is provided a transgenic non-human animal having a transgene that expresses a Glutamate Transporter Associated Protein chromosomally integrated into the germ cells of the animal. An embodiment of the invention provides a method for producing such transgenic animals.

In another embodiment of the invention, there is provided a computer readable medium having stored thereon a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 and sequences substantially identical thereto, or a polypeptide sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 and sequences substantially identical thereto.

In another embodiment of the invention, there is provided a computer system comprising a processor and a data storage device wherein said data storage device has stored thereon a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 and sequences substantially identical thereto, or a polypeptide sequence selected from the group consisting SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 and sequences substantially identical thereto.

In yet another embodiment of the invention, there is provided a method for comparing a first sequence to a reference sequence wherein said first sequence is a nucleic acid sequence selected from the group consisting SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 and sequences substantially identical thereto, or a polypeptide sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID

NO:6 and sequences substantially identical thereto. The method comprises reading the first sequence and the reference sequence through use of a computer program which compares sequences, and determining differences between the first sequence and the reference sequence with the computer program.

In yet another embodiment of the invention there is provided a method for identifying a feature in a sequence wherein the sequence is selected from the group consisting of a nucleic acid sequence SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 sequences substantially identical thereto, or a polypeptide sequence SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 and sequences substantially identical thereto. The method includes reading the sequence through the use of a computer program which identifies features in sequences and identifying features in the sequences with the computer program.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show schematic representations of GTRAP4-41 and GTRAP4-48, respectively.

Figure 2 shows overlapping deletion mutants of the carboxy terminus of EAAT4 used to identify domains interacting with GTRAP4-41 and GTRAP4-48.

Figure 3A shows the effect of GTRAP4-41 and GTRAP4-48 on sodium-dependent glutamate uptake in transfected HEK-rEAAT4 cells. **Figure 3B** shows kinetic data which demonstrates that GTRAP4-41, in the presence of EAAT4, increases the V_{max} of glutamate uptake.

Figure 4A shows the effect of GTRAP3-18 on sodium-dependent glutamate transport in transfected HEK-293 cells. **Figure 4B** shows that the effect of GTRAP 3-18 on EAAC1-mediated glutamate transport is specific.

Figures 5A and 5B show the effect of Glutamate Transporter Associated Proteins (GTRAPs) on glutamate transporter protein expression.

Figures 6A-C show the effect of GTRAPs on glutamate transporter protein activity.

Figures 7A and 7B show the interaction between GTRAP4-48 and RhoGEF.

Figures 8A-C show the effects of GTRAP3-18 antisense oligonucleotide on glutamate transport.

Figures 9A-E show the effect of retinoic acid on GTRAP3-18-mediated glutamate transport.

Figure 10 is a flow diagram illustrating a computer system, data retrieving device and display.

Figure 11 is a flow diagram illustrating one embodiment of process 200 for comparing a new nucleotide or protein sequence with a database of sequences in order to determine the homology levels between the new sequence and the sequences in the database.

Figure 12 is a flow diagram illustrating one embodiment of a process 250 in a computer for determining whether two sequences are homologous.

Figure 13 is a flow diagram illustrating one embodiment of a process 300 for comparing features in polynucleotide and polypeptide sequences.

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Figure 14 (A-C) shows a nucleic acid sequence of a polynucleotide encoding GTRAP4-41.

Figure 15 shows an amino acid sequence of GTRAP4-41.

Figure 16 (A and B) shows a nucleic acid sequence of a polynucleotide encoding GTRAP4-48.

Figure 17 shows an amino acid sequence of GTRAP4-48.

Figure 18 shows a nucleic acid sequence of a polynucleotide encoding GTRAP3-18.

Figure 19 shows an amino acid sequence of GTRAP3-18.

Figure 20 (A and B) shows a nucleic acid sequence of a polynucleotide encoding PCTAIRE-1.

Figure 21 shows an amino acid sequence of PCTAIRE-1a.

Figure 22 shows an amino acid sequence of PCTAIRE1b.

DETAILED DESCRIPTION OF THE INVENTION

The identification of molecules regulating the transport of neurotransmitters is central to understanding the mechanisms of neural activity, synaptic plasticity and learning. Efficient and rapid removal of neurotransmitters from the synaptic cleft by neurotransmitter transporters is critical to synaptic transmission. Re-uptake of glutamate by glutamate transporters both terminates the synaptic action of glutamate, thereby preventing glutamate-mediated exotoxicity and recaptures glutamate molecules for possible reuse.

Accordingly, one embodiment of the invention provides a substantially pure polypeptide characterized as modulating intracellular glutamate transport, interacting with a glutamate transporter protein and having an expression pattern in the brain. A polypeptide molecule having such characteristics is known as a Glutamate Transporter Associated Protein (GTRAP). Glutamate Transporter Associated Proteins can be further characterized as having at least one PDZ domain, having at least one regulatory G-protein domain, having at least one pleckstrin homology domain, having at least one proline-rich domain, and having at least one guanine exchange factor domain. Glutamate Transporter Associated Protein can also be characterized as having at least one pleckstrin homology domain, having at least one spectrin repeat, and having at least one α-actinin domain.

Glutamate Transporter Associated Proteins modulate glutamate transport. Glutamate transport refers to the active movement of glutamate across a cellular membrane. Glutamate transport is an essential component of central nervous system glutamatergic neurotransmission. For example, glutamate transport is essential in the inactivation of synaptically released glutamate and the prevention of excitotoxicity. The concentration of glutamate is higher in the terminal than in the synaptic cleft, even following neurotransmitter release. Nonetheless, the transporters take up glutamate from the synaptic cleft and transport it into the cell. Glutamate transporters also serve to bring glutamate into the cell for use in cellular metabolism, e.g. provide glutamate for new synthesis of neurotransmitter GABA. GTRAPs associated with some types of glutamate transporter protein, for example, glutamate transporter protein EAAT4, stimulate glutamate transport. GTRAPs associated with other types of glutamate transporter proteins, for example, EAAC1, inhibit glutamate transport.

While not wishing to be bound to any one mechanism, the modulation in transport appears to be effected through a change in Vmax or a change in Km (see Examples section). Glutamate transporter proteins can signal messages to the cell about transport activities e.g. GTRAP48 activate G-protein signaling].

Glutamate Transporter Associated Proteins share several common features. All GTRAPs are able to interact with at least one glutamate transporter protein. Glutamate transporter proteins include GLAST, GLT-1, EAAC1, EAAT1, EAAT2, EAAT3, EAAT4 and EAAT5. Glutamate transporters share over 50% amino acid sequence identity with each other, and display almost identical hydrophobic profiles including six prominent hydrophobic peaks, followed by a small hydrophobic peak and long hydrophobic stretch. The proteins are generally 500 to 600 amino acids in length, with high conservation of sequence in the transmembrane domain. The carboxyl and amino terminal domains are intracellular and have the least sequence conservation among all transporters. Less is known about the genomic structure of the transporter proteins. The glutamate transporter family is quite distinct in structure from the 12 transmembrane α-helix arrangement of another sodium- and chloride-dependent transporter family related to dopamine and serotonin transport. The glutamate transporter family transports L-glutamate, D-aspartate and L-aspartate and some other acidic amino acids such as threo-β-hydroxyaspartate (THA) and cysteate. However, the transporters display distinct properties in substrate or inhibitor selectivity, e.g. dihydrokainate is a specific inhibitor of GLT-1 and EAAC1 transports cysteine with much higher affinity than the other transporters. Various studies have suggested that transporters may form homomultimers, perhaps dimers, but physiological transport may only require monomers of the protein.

Immunohistochemical studies show that GLAST and GLT-1 (EAAT1 and EAAT2) are localized primarily in astrocytes. In the adult CNS, GLT-1 is widely distributed throughout the brain and spinal cord in astroglial cell bodies and processes, while GLAST protein is localized in glial cells of cerebellar molecular and granule cell layers, and in some astroglia throughout the brain . Double labeling post-embedding electron microscopic immunocytochemistry shows the two glial transporters, GLT-1 and GLAST, expressed in the same cell membrane. Each protein forms oligomeric complexes but GLT-1 and GLAST may not complex with each

other. Antisense knock-down studies show that these two glial transporters are responsible for over 80% of glutamate uptake in the brain, an observation later confirmed in GLT-1 null mice. Quantitative immunoblotting and electron microscopy indicate that the glial transporters are quite abundant; GLAST and GLT-1 respectively, are 2300 and 8500 molecules per μm^2 in CA1 hippocampus membrane, and 4700 and 740 molecules per μm^2 in the cerebellar molecular layer.

Developmental studies reveal differential expression of GLT-1 and GLAST mRNA and protein. Initially expression of GLAST predominates throughout the CNS, followed by a shift in expression to the cerebellum, whereas GLT-1 expression remains throughout most of the CNS. A dramatic up-regulation of GLT-1 gene expression at post-natal day 14 coincides with the post-natal development of glutamatergic transmission in the cortex.

GLT-1 mRNA and protein can, under certain conditions be found in neurons, *e.g.* cultured hippocampal neurons. Transiently localized GLT-1 on growing axons and axon pathways can also be detected. Additional studies in models of ischemic brain injury and in fetal ovine brain suggest rare neuronal expression of GLT-1 as well.

EAAC1 and EAAT4 are neuronal transporters. EAAC1 immunoreactivity is particularly high in regions such as the hippocampus, cerebellum and basal ganglia. It is widely distributed in neurons such as large cortical pyramidal neurons, and is also present in non-glutamatergic neurons including GABAergic cerebellar Purkinje cells. Ultra-structural studies suggest that EAAC1 is not a presynaptic transporter of glutamatergic neurons. In fact, EAAC1 appears to be primarily localized in the somatodendritic compartment, and is already expressed at stages preceding synaptic contact formation. Rarely, EAAC1 is found in pre-synaptic terminals, which are always inhibitory (e.g GABAergic). Ultra structurally, EAAC1 is present in dendrites and somas. Detailed EM -gold studies of synapses indicate that the protein is most often peri-synaptic in location, like EAAT4. EAAC1 is also widely expressed outside the central nervous system, so it may serve metabolic functions in neurons. For example, it may provide glutamate for resynthesis of GABA in GABAergic terminals, where the protein has been localized (Rothstein, *et al.* (1994) Neuron 13:713-725, herein incorporated by reference in its entirety). In fact, studies using

antisense oligonucleotides to inhibit EAAC1 suggest that this transporter may, in part, regulate GABA synthesis.

EAAT4 is largely expressed in the cerebellum with very faint levels of expression in hippocampus, neocortex, striatum, brain stem and thalamus, in both the adult human and rat CNS . EAAT4 is present at low concentrations in the synaptic membrane, but is highly enriched in the parts of the dendritic and spine membranes facing astrocytes. A functional relationship may exist between EAAT4 and the glial transporters, and that EAAT4, having a prominent Cl^--channel property, may function as a combined transporter and inhibitory glutamate receptor. The average density of EAAT4 protein in the Purkinje cell membrane has been calculated to be 1800 molecules per μ^2. Immunohistochemical as well as immunoblot analysis demonstrates that during development EAAT4 protein is expressed in the human cerebellum both pre- and post-natally, while its expression in the frontal cortex is restricted to fetal stages. In the cerebellum, Purkinje cells show faint EAAT4 immunoreactivity at gestation week 17. However, EAAT4 expression becomes increasingly intense from gestation week 23 to the infantile period. After the late infantile period, EAAT4 immunoreactivity shows the same pattern as in adults. The intracellular localization of EAAT4 also changes with development. In the early embryonic period, EAAT immunoreactivity is found in the short processes of the Purkinje cells, while in the late fetal to early infantile periods, EAAT4 immunoreactivity is found in the cell bodies and dendrites, and in the late infantile period, it is found in the spines.

Glutamate transporters and glutamate receptors are compartmentalized in and around the synaptic cleft and proteins capable of glutamate receptor membrane targeting and the epitopes responsible for these events are known. For example, three cytoplasmic molecules have been recently identified which bind to the final eight amino acids in the C-terminus of GluR2 and GluR3, but not to GluR1 or NR1. These molecules, named GRIP and ABP are all synaptically localized in the hippocampus and contain one or more PDZ domains, protein binding motifs of between 70 and 90 amino acids which have recently been implicated in the localization of other highly regulated proteins. None of these molecules interacts or regulates glutamate transporters.

Several studies document a role for neurons in modulating the expression and activity of glutamate transporters. Pathway lesion studies suggest that neurons can influence the astroglial (but not neuronal) expression of glutamate transporter subtypes. This has been validated in vitro, where astroglial (EAAT2) expression in cultured astrocytes appears to depend on neurons, most likely secreted factors, including glutamate itself. In fact, a number of trophic factors that modulate EAAT2 expression in various in vitro preparations have been identified. Protein kinase C phosphorylation of EAAT2 (GLT-1) has also been found to stimulate transport. Transporters can also be directly regulated through other signaling pathways. Activity of EAAC1 (and GABA and serotonin transporters) can be regulated through expression at the cell surface, via regulated cellular trafficking, occurring in part through protein kinase C and phosphatidylinositol 3-kinase pathways.

Glutamate Transporter Associated Proteins have an expression pattern in brain tissue. Immunofluorescence staining of brain tissue reveals a pattern of GTRAP immunoreactivity in brain tissue. Prominent immunolocalization is observed in the cerebellar cortex, especially in Purkinje cell somas and dendrites with no axonal localization. Expression is also observed in other brain regions including striatum, hippocampus and thalamus.

Expression of certain Glutamate Transporter Associated Proteins is observed outside the brain. For example, GTRAP3-18 is expressed in the liver, kidney, heart, muscle as well as in the central nervous system.

Glutamate Transporter Associated Proteins can include at least one PDZ domain, at least one regulatory G-protein domain, at least one pleckstrin homology domain (PH), at least one proline-rich domain, at least one guanine exchange factor domain (Dbl), at least one spectrin repeat and at least one α-actinin domain. Methods to identify such domains are known to those of skill in the art. For example, computer programs that compare invention nucleic acid and amino acid sequences to nucleic acid and amino acid sequences, and identify regions of homology can be used to identify such domains.

Exemplary Glutamate Transporter Associated Proteins of the invention include sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 and

conservative variants thereof. The terms "conservative variation" and "substantially similar" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine, and the like. The terms "conservative variation" and "substantially similar" also include the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

Also contemplated by the invention are polypeptides that share at least 90% sequence homology to the polypeptide sequences set forth as SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6. Sequence homology can be determined by those of skill in the art, for example, by computer programs that compare sequences such as Blast.

Exemplary polynucleotides encoding a Glutamate Transporter Associated Proteins are set forth as SEQ ID NO: 1, SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7. The term "polynucleotide", "nucleic acid", "nucleic acid sequence", or "nucleic acid molecule" refers to a polymeric form of nucleotides at least 10 bases in length. By "isolated polynucleotide" is meant a polynucleotide that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (*e.g.*, a cDNA) independent of other sequences. The nucleotides of the invention can be deoxyribonucleotides, ribonucleotides in which uracil (U) is present in place of thymine (T), or modified forms of either nucleotide. The nucleotides of the invention can be complementary to the deoxynucleotides or to the ribonucleotides. A polynucleotide encoding a Glutamate Transporter Associated Protein includes "degenerate variants", sequences that are degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one

codon. Therefore, all degenerate nucleotide sequences are included in the invention as long as the amino acid sequence of a polypeptide encoded by the nucleotide sequence of SEQ ID NO: 1,SEQ ID NO:3, SEQ ID NO:5 or SEQ ID NO:7 is functionally unchanged.

A nucleic acid molecule encoding a Glutamate Transporter Associated Protein includes sequences encoding functional Glutamate Transporter Associated Protein polypeptides as well as functional fragments thereof. As used herein, the term "functional polypeptide" refers to a polypeptide which possesses biological function or activity which is identified through a defined functional assay, and which is associated with a particular biologic, morphologic, or phenotypic alteration in the cell. The term "functional fragments of Glutamate Transporter Associated Protein," refers to fragments of a Glutamate Transporter Associated Protein that retain a Glutamate Transporter Associated Protein activity, *e.g.*, the ability to interact with a glutamate transporter protein, modulate intracellular glutamate transport, and the like. Additionally, functional Glutamate Transporter Associated Protein fragments may act as competitive inhibitors of Glutamate Transporter Associated Protein binding to a glutamate transporter protein, for example. Biologically functional fragments can vary in size from a polypeptide fragment as small as an epitope capable of binding an antibody molecule to a large polypeptide capable of participating in the characteristic induction or programming of phenotypic changes within a cell. Nucleotide fragments of the invention have at least 15 base pairs and hybridize to a polynucleotide encoding a polypeptide as set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:22.

Further embodiments of the invention provide isolated polynucleotides, wherein the nucleotide is at least 15 base pairs in length which hybridizes under moderately to highly stringent conditions to DNA encoding a polypeptide as set forth in SEQ ID NO:2 or to DNA encoding a polypeptide as set forth in SEQ ID NO:4, or SEQ ID NO:6. In nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (*e.g.*, GC v. AT content), and nucleic acid type (*e.g.*, RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting

hybridization conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter.

An example of progressively higher stringency conditions is as follows: 2 x SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2 x SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2 x SSC/0.1% SDS at about 42°C (moderately stringent conditions); and 0.1 x SSC at about 68°C (highly stringent conditions). Washing can be carried out using only one of these conditions, *e.g.*, high stringency conditions, or each of the conditions can be used, *e.g.*, for 10-15 minutes each, in the order listed above, repeating any or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the particular hybridization reaction involved, and can be determined empirically.

Antibodies of the invention may bind to Glutamate Transporter Associated Proteins provided by the invention to prevent normal interactions of Glutamate Transporter Associated Proteins. Binding of antibodies to Glutamate Transporter Associated Protein can interfere with for example, glutamate transport, with cytoskeletal stability by interfering with intracellular protein binding, with expression patterns of Glutamate Transporter Associated Proteins or with interactions with glutamate transporter proteins . Furthermore, binding of antibodies to Glutamate Transporter Associated Proteins can interfere with the localization of glutamate transporter proteins on cellular membranes.

The antibodies of the invention can be used in any subject in which it is desirable to administer *in vitro* or *in vivo* immunodiagnosis or immunotherapy. The antibodies of the invention are suited for use, for example, in immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier. In addition, the antibodies in these immunoassays can be detectably labeled in various ways. Examples of types of immunoassays which can utilize antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA), the enzyme-linked immunosorbant assay (ELISA) and the sandwich (immunometric) assay. Detection of the antigens using the antibodies of the invention can be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. Those of skill in the art will

know, or can readily discern, other immunoassay formats without undue experimentation.

The term "antibody" as used in this invention includes intact molecules as well as fragments thereof, such as Fab, F(ab')2, and Fv which are capable of binding to an epitopic determinant present in an invention polypeptide. Such antibody fragments retain some ability to selectively bind with its antigen or receptor.

Methods of making these fragments are known in the art. (See for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1988), incorporated herein by reference). Monoclonal antibodies are made from antigen containing fragments of the protein by methods well known to those skilled in the art (Kohler & Milstein, *Nature* 256:495 (1975); Coligan *et al.*, sections 2.5.1-2.6.7; and Harlow *et al.*, *Antibodies: A Laboratory Manual*, page 726 (Cold Spring Harbor Pub. 1988), which are hereby incorporated by reference. Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen/ligand, verifying the presence of antibody production by analyzing a serum sample, removing the spleen to obtain B lymphocytes, fusing the B lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures. Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. *See, e.g.*, Coligan *et al.*, sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3; Barnes *et al.*, "Purification of Immunoglobulin G (IgG)" in *Methods In Molecular Biology*, VOL. 10, pages 79-104 (Humana Press 1992).

Antibodies which bind to an invention Glutamate Transporter Associated Protein polypeptide can be prepared using an intact polypeptide or fragments containing small peptides of interest as the immunizing antigen. For example, it may be desirable to produce antibodies that specifically bind to the amino- or carboxyl-terminal domains of an invention polypeptide. For the preparation of polyclonal antibodies, the polypeptide or peptide used to immunize an animal is derived from translated cDNA or chemically synthesized and can be conjugated to a carrier protein,

if desired. Commonly used carrier proteins which may be chemically coupled to the immunizing peptide include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), tetanus toxoid, and the like.

Invention polyclonal or monoclonal antibodies can be further purified, for example, by binding to and elution from a matrix to which the polypeptide or a peptide to which the antibodies were raised is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies (See, for example, Coligan, *et al.*, Unit 9, Current Protocols in Immunology, Wiley Interscience, 1994, incorporated herein by reference).

The antibodies of the invention can be bound to many different carriers and used to detect the presence of an antigen comprising the polypeptides of the invention. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention . Those skilled in the art will know of other suitable carriers for binding antibodies, or will be able to ascertain such, using routine experimentation.

There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, phosphorescent compounds, and bioluminescent compounds. Those of ordinary skill in the art will know of other suitable labels for binding to the antibody, or will be able to ascertain such, using routine experimentation.

Another technique which may also result in greater sensitivity consists of coupling the antibodies to low molecular weight haptens. These haptens can then be specifically detected by means of a second reaction. For example, it is common to use such haptens as biotin, which reacts with avidin, or dinitrophenyl, puridoxal, and fluorescein, which can react with specific antihapten antibodies.

In using the monoclonal and polyclonal antibodies of the invention for the in vivo detection of antigen, *e.g.*, a Glutamate Transporter Associated Protein, the detectably labeled antibody is given a dose which is diagnostically effective. The term "diagnostically effective" means that the amount of detectably labeled antibody is administered in sufficient quantity to enable detection of the site having the antigen comprising a polypeptide of the invention for which the antibodies are specific.

The concentration of detectably labeled antibody which is administered should be sufficient such that the binding to those cells having the polypeptide is detectable compared to the background. Further, it is desirable that the detectably labeled antibody be rapidly cleared from the circulatory system in order to give the best target-to-background signal ratio.

As a rule, the dosage of detectably labeled antibody for in vivo treatment or diagnosis will vary depending on such factors as age, sex, and extent of disease of the individual. Such dosages may vary, for example, depending on whether multiple injections are given, antigenic burden, and other factors known to those of skill in the art.

A polynucleotide agent can be contained in a vector, which can facilitate manipulation of the polynucleotide, including introduction of the polynucleotide into a target cell. The vector can be a cloning vector, which is useful for maintaining the polynucleotide, or can be an expression vector, which contains, in addition to the polynucleotide, regulatory elements useful for expressing the polynucleotide and, where the polynucleotide encodes a peptide, for expressing the encoded peptide in a particular cell. An expression vector can contain the expression elements necessary to achieve, for example, sustained transcription of the encoding polynucleotide, or the regulatory elements can be operatively linked to the polynucleotide prior to its being cloned into the vector.

An expression vector (or the polynucleotide) generally contains or encodes a promoter sequence, which can provide constitutive or, if desired, inducible or tissue specific or developmental stage specific expression of the encoding polynucleotide, a poly-A recognition sequence, and a ribosome recognition site or internal ribosome entry site, or other regulatory elements such as an enhancer, which can be tissue

specific. The vector also can contain elements required for replication in a prokaryotic or eukaryotic host system or both, as desired. Such vectors, which include plasmid vectors and viral vectors such as bacteriophage, baculovirus, retrovirus, lentivirus, adenovirus, vaccinia virus, semliki forest virus and adeno-associated virus vectors, are well known and can be purchased from a commercial source (Promega, Madison WI; Stratagene, La Jolla CA; GIBCO/BRL, Gaithersburg MD) or can be constructed by one skilled in the art (see, for example, Meth. Enzymol., Vol. 185, Goeddel, ed. (Academic Press, Inc., 1990); Jolly, Canc. Gene Ther. 1:51-64, 1994; Flotte, J. Bioenerg. Biomemb. 25:37-42, 1993; Kirshenbaum et al., J. Clin. Invest. 92:381-387, 1993; each of which is incorporated herein by reference).

A polynucleotide useful in a method of the invention also can be operatively linked to tissue specific regulatory element, for example, a neuron specific regulatory element, such that expression of an encoded peptide agent is restricted to neurons in an individual, or to neurons in a mixed population of cells in culture, for example, an organ culture. For example, neuronal or glial promoters such as the myelin basic protein promoter, other neuronal-specific promoters, and astroglial promoters (e.g. GFAP- glial fibrillary acidic protein), known to those of skill in the art may be used. Muscle-regulatory elements including, for example, the muscle creatine kinase promoter (Sternberg et al., Mol. Cell. Biol. 8:2896-2909, 1988, which is incorporated herein by reference) and the myosin light chain enhancer/promoter (Donoghue et al., Proc. Natl. Acad. Sci., *USA* 88:5847-5851, 1991, which is incorporated herein by reference) are well known in the art. A variety of other promoters have been identified which are suitable for up regulating expression in cardiac tissue. Included, for example, are the cardiac I-myosin heavy chain (AMHC) promoter and the cardiac I-actin promoter. Other examples of tissue-specific regulatory elements include, tissue-specific promoters, pancreatic (insulin or elastase), and actin promoter in smooth muscle cells. Through the use of promoters, such as milk-specific promoters, recombinant retroviruses may be isolated directly from the biological fluid of the progeny.

A Glutamate Transporter Associated Protein polynucleotide of the invention can be inserted into a vector, which can be a cloning vector or a recombinant

expression vector. The term "expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of a polynucleotide, particularly, with respect to the present invention, a polynucleotide encoding all or a peptide portion of a Glutamate Transporter Associated Protein. Such expression vectors contain a promoter sequence, which facilitates the efficient transcription of the inserted genetic sequence of the host. The expression vector generally contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells. Vectors suitable for use in the present invention include, but are not limited to, the T7-based expression vector for expression in bacteria (Rosenberg, et al., Gene 56:125, 1987), the pMSXND expression vector for expression in mammalian cells (Lee and Nathans, J. Biol. Chem. 263:3521, 1988) and baculovirus-derived vectors for expression in insect cells. The DNA segment can be present in the vector operably linked to regulatory elements, for example, a promoter, which can be a T7 promoter, metallothionein I promoter, polyhedrin promoter, or other promoter as desired, particularly tissue specific promoters or inducible promoters.

Viral expression vectors can be particularly useful for introducing a polynucleotide useful in a method of the invention into a cell, particularly a cell in a subject. Viral vectors provide the advantage that they can infect host cells with relatively high efficiency and can infect specific cell types. For example, a polynucleotide encoding a Glutamate Transporter Associated Protein or functional peptide portion thereof can be cloned into a baculovirus vector, which then can be used to infect an insect host cell, thereby providing a means to produce large amounts of the encoded protein or peptide portion. The viral vector also can be derived from a virus that infects cells of an organism of interest, for example, vertebrate host cells such as mammalian, avian or piscine host cells. Viral vectors can be particularly useful for introducing a polynucleotide useful in performing a method of the invention into a target cell. Viral vectors have been developed for use in particular host systems, particularly mammalian systems and include, for example, retroviral vectors, other lentivirus vectors such as those based on the human immunodeficiency virus (HIV), adenovirus vectors, adeno-associated virus vectors, herpesvirus vectors, vaccinia virus vectors, and the like (see Miller and Rosman, BioTechniques 7:980-990, 1992; Anderson et al., Nature 392:25-30 Suppl., 1998; Verma and Somia, Nature

389:239-242, 1997; Wilson, New Engl. J. Med. 334:1185-1187 (1996), each of which is incorporated herein by reference).

When retroviruses, for example, are used for gene transfer, replication competent retroviruses theoretically can develop due to recombination of retroviral vector and viral gene sequences in the packaging cell line utilized to produce the retroviral vector. Packaging cell lines in which the production of replication competent virus by recombination has been reduced or eliminated can be used to minimize the likelihood that a replication competent retrovirus will be produced. All retroviral vector supernatants used to infect cells are screened for replication competent virus by standard assays such as PCR and reverse transcriptase assays. Retroviral vectors allow for integration of a heterologous gene into a host cell genome, which allows for the gene to be passed to daughter cells following cell division.

A polynucleotide, which can be contained in a vector, can be introduced into a cell by any of a variety of methods known in the art (Sambrook et al., Molecular Cloning: A laboratory manual (Cold Spring Harbor Laboratory Press 1989); Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, MD (1987, and supplements through 1995), each of which is incorporated herein by reference). Such methods include, for example, transfection, lipofection, microinjection, electroporation and, with viral vectors, infection; and can include the use of liposomes, microemulsions or the like, which can facilitate introduction of the polynucleotide into the cell and can protect the polynucleotide from degradation prior to its introduction into the cell. The selection of a particular method will depend, for example, on the cell into which the polynucleotide is to be introduced, as well as whether the cell is isolated in culture, or is in a tissue or organ in culture or *in situ*.

Introduction of a polynucleotide into a cell by infection with a viral vector is particularly advantageous in that it can efficiently introduce the nucleic acid molecule into a cell *ex vivo* or *in vivo* (see, for example, U.S. Patent No. 5,399,346, which is incorporated herein by reference). Moreover, viruses are very specialized and can be selected as vectors based on an ability to infect and propagate in one or a few specific cell types. Thus, their natural specificity can be used to target the nucleic acid molecule contained in the vector to specific cell types. As such, a vector based on an

HIV can be used to infect T cells, a vector based on an adenovirus can be used, for example, to infect respiratory epithelial cells, a vector based on a herpesvirus can be used to infect neuronal cells, and the like. Other vectors, such as adeno-associated viruses can have greater host cell range and, therefore, can be used to infect various cell types, although viral or non-viral vectors also can be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

A polynucleotide sequence encoding a Glutamate Transporter Associated Protein can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect and mammalian organisms. Methods of expressing polynucleotides having eukaryotic or viral sequences in prokaryotes are well known in the art, as are biologically functional viral and plasmid DNA vectors capable of expression and replication in a host. Methods for constructing an expression vector containing a polynucleotide of the invention are well known, as are factors to be considered in selecting transcriptional or translational control signals, including, for example, whether the polynucleotide is to be expressed preferentially in a particular cell type or under particular conditions (see, for example, Sambrook et al., *supra*, 1989).

A variety of host cell/expression vector systems can be utilized to express a Glutamate Transporter Associated Protein coding sequence, including, but not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors; yeast cells transformed with recombinant yeast expression vectors; plant cell systems infected with recombinant virus expression vectors such as a cauliflower mosaic virus or tobacco mosaic virus, or transformed with recombinant plasmid expression vector such as a Ti plasmid; insect cells infected with recombinant virus expression vectors such as a baculovirus; animal cell systems infected with recombinant virus expression vectors such as a retrovirus, adenovirus or vaccinia virus vector; and transformed animal cell systems genetically engineered for stable expression. Where the expressed Glutamate Transporter Associated Protein is post-translationally modified, for example, by glycosylation, it can be particularly advantageous to select a host cell/expression vector system that can effect the desired modification, for example, a mammalian host cell/expression vector system.

Depending on the host cell/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, and the like can be used in the expression vector (Bitter et al., Meth. Enzymol. 153:516-544, 1987). For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage Σ, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like can be used. When cloning in mammalian cell systems, promoters derived from the genome of mammalian cells, for example, a human or mouse metallothionein promoter, or from mammalian viruses, for example, a retrovirus long terminal repeat, an adenovirus late promoter or a vaccinia virus 7.5K promoter, can be used. Promoters produced by recombinant DNA or synthetic techniques can also be used to provide for transcription of the inserted GDF receptors coding sequence.

In yeast cells, a number of vectors containing constitutive or inducible promoters can be used (see Ausubel et al., *supra*, 1987, see chapter 13; Grant et al., Meth. Enzymol. 153:516-544, 1987; Glover, DNA Cloning Vol. II (IRL Press, 1986), see chapter 3; Bitter, Meth. Enzymol. 152:673-684, 1987; see, also, The Molecular Biology of the Yeast *Saccharomyces* (Eds., Strathern et al., Cold Spring Harbor Laboratory Press, 1982), Vols. I and II). A constitutive yeast promoter such as ADH or LEU2 or an inducible promoter such as GAL can be used (Rothstein, DNA Cloning Vol. II (*supra*, 1986), chapter 3). Alternatively, vectors can be used which promote integration of foreign DNA sequences into the yeast chromosome.

Eukaryotic systems, particularly mammalian expression systems, allow for proper post-translational modifications of expressed mammalian proteins. Eukaryotic cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, phosphorylation, and advantageously, plasma membrane insertion of the gene product can be used as host cells for the expression of a Glutamate Transporter Associated Protein, or functional peptide portion thereof.

Mammalian cell systems which utilize recombinant viruses or viral elements to direct expression can be engineered. For example, when using adenovirus expression vectors, the Glutamate Transporter Associated Protein coding sequence can be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. Alternatively, the vaccinia virus 7.5K

promoter can be used (Mackett et al., Proc. Natl. Acad. Sci., *USA* 79:7415-7419, 1982; Mackett et al., J. Virol. 49:857-864, 1984; Panicali et al., Proc. Natl. Acad. Sci., *USA* 79:4927-4931, 1982). Particularly useful are bovine papilloma virus vectors, which can replicate as extrachromosomal elements (Sarver et al., Mol. Cell. Biol. 1:486, 1981). Shortly after entry of this DNA into mouse cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid into the host cell chromosome, thereby yielding a high level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as, for example, the neo gene. Alternatively, the retroviral genome can be modified for use as a vector capable of introducing and directing the expression of the Glutamate Transporter Associated Protein gene in host cells (Cone and Mulligan, Proc. Natl. Acad. Sci., *USA* 81:6349-6353, 1984). High level expression can also be achieved using inducible promoters, including, but not limited to, the metallothionein IIA promoter and heat shock promoters.

For long term, high yield production of recombinant proteins, stable expression is preferred. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with Glutamate Transporter Associated Protein cDNA controlled by appropriate expression control elements such as promoter, enhancer, sequences, transcription terminators, and polyadenylation sites, and a selectable marker. The selectable marker in the recombinant plasmid can confer resistance to the selection, and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci, which, in turn can be cloned and expanded into cell lines. For example, following the introduction of foreign DNA, engineered cells can be allowed to grow for 1 to 2 days in an enriched media, and then are switched to a selective media. A number of selection systems can be used, including, but not limited to, the herpes simplex virus thymidine kinase (Wigler et al., Cell 11:223, 1977), hypoxanthine-guanine phosphoribosyltransferase (Szybalska and Szybalski, Proc. Natl. Acad. Sci., *USA* 48:2026, 1982), and adenine phosphoribosyltransferase (Lowy, et al., Cell 22:817, 1980) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., Proc. Natl. Acad. Sci. *USA* 77:3567, 1980; O'Hare et al., Proc. Natl. Acad. Sci., *USA* 78: 1527, 1981); gpt, which confers resistance to mycophenolic acid (Mulligan and

Berg, Proc. Natl. Acad. Sci., *USA* 78:2072, 1981); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., J. Mol. Biol. 150:1, 1981); and hygro, which confers resistance to hygromycin (Santerre et al., Gene 30:147, 1984) genes. Additional selectable genes, including trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman and Mulligan, Proc. Natl. Acad. Sci., *USA* 85:8047, 1988); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, Curr. Comm. Mol. Biol. (Cold Spring Harbor Laboratory Press, 1987), also have been described.

When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate coprecipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors can be used. Eukaryotic cells can also be cotransformed with DNA sequences encoding Glutamate Transporter Associated Proteins of the invention, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein. (Gluzman, Eukaryotic Viral Vectors (Cold Spring Harbor Laboratory Press, 1982)).

The invention provides a method for producing a polypeptide characterized as interacting with a glutamate transporter protein; modulating intracellular glutamate transport; having an expression pattern in Purkinje cells of the brain; and being hydrophobic. The invention also provides a method for producing a polypeptide encoded by the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:6 or fragments thereof, including culturing the host cell under conditions suitable for the expression of the polypeptide and recovering the polypeptide from the host cell culture.

A Glutamate Transporter Associated Protein polypeptide or a fragment thereof, can be encoded by a recombinant or non-recombinant nucleic acid molecule and expressed in a cell. Preparation of a Glutamate Transporter Associated Protein polypeptide by recombinant methods provides several advantages. In particular, the

nucleic acid sequence encoding the Glutamate Transporter Associated Protein polypeptide can include additional nucleotide sequences encoding, for example, peptides useful for recovering the Glutamate Transporter Associated Protein polypeptide from the host cell. A Glutamate Transporter Associated Protein polypeptide can be recovered using well known methods, including, for example, precipitation, gel filtration, ion exchange, reverse-phase, or affinity chromatography (see, for example, Deutscher et al., "Guide to Protein Purification" in Meth. Enzymol., Vol. 182, (Academic Press, 1990)). Such methods also can be used to purify a fragment of a Glutamate Transporter Associated Protein polypeptide, for example, a particular binding sequence, from a cell in which it is naturally expressed.

A recombinant nucleic acid molecule encoding a Glutamate Transporter Associated Protein polypeptide or a fragment thereof can include, for example, a protease site, which can facilitate cleavage of the Glutamate Transporter Associated Protein polypeptide from a non-Glutamate Transporter Associated Protein polypeptide sequence, for example, a tag peptide, secretory peptide, or the like. As such, the recombinant nucleic acid molecule also can encode a tag peptide such as a polyhistidine sequence, a FLAG peptide (Hopp et al., Biotechnology 6:1204 (1988)), a glutathione S-transferase polypeptide or the like, which can be bound by divalent metal ions, a specific antibody (U.S. Patent No. 5,011,912), or glutathione, respectively, thus facilitating recovery and purification of the Glutamate Transporter Associated Protein polypeptide comprising the peptide tag. Such tag peptides also can facilitate identification of the Glutamate Transporter Associated Protein polypeptide through stages of synthesis, chemical or enzymatic modification, linkage, or the like. Methods for purifying polypeptides comprising such tags are well known in the art and the reagents for performing such methods are commercially available.

A nucleic acid molecule encoding a Glutamate Transporter Associated Protein polypeptide can be engineered to contain one or more restriction endonuclease recognition and cleavage sites, which can facilitate, for example, substitution of an element of the Glutamate Transporter Associated Protein polypeptide such as the selective recognition domain or, where present, a spacer element. As such, related Glutamate Transporter Associated Protein polypeptides can be prepared, each having a similar activity, but having specificity for different function-forming contexts. A

restriction endonuclease site also can be engineered into (or out of) the sequence coding a peptide portion of the Glutamate Transporter Associated Protein polypeptide, and can, but need not change one or more amino acids encoded by the particular sequence. Such a site can provide a simple means to identify the nucleic acid sequence, based on cleavage (or lack of cleavage) following contact with the relevant restriction endonuclease, and, where introduction of the site changes an amino acid, can further provide advantages based on the substitution.

In another embodiment of the invention there is provided a substantially pure polypeptide which interacts with amino acid sequence QEAELTLP (SEQ ID NO:9) or the amino acid sequence GRGGNESVM (SEQ ID NO:10). In a preferred embodiment, polypeptides interact with a Glutamate Transporter Associated Protein encoded by a polynucleotide that hybridizes to SEQ ID NO:1. An exemplary protein containing amino acid sequences QEAELTLP (SEQ ID NO:9) and GRGGNESVM (SEQ ID NO:10) is glutamate transport protein EAAT4 (see Examples). In another embodiment of the invention, there is provided a polynucleotide encoding a substantially pure polypeptide which interacts with amino acid sequence QEAELTLP (SEQ ID NO:9) or the amino acid sequence GRGGNESVM (SEQ ID NO:10).

Another embodiment of the invention provides a substantially pure polypeptide which interacts with a polypeptide having the sequence of amino acids found at amino acid residues 527 to 534 of EAAT4 (SEQ ID NO:9). Still another embodiment of the invention provides a substantially pure polypeptide which interacts with a polypeptide sequence having the sequence of amino acid found at amino acid residues 555 to 561 of EAAT4 (SEQ ID NO:10).

Still another embodiment of the invention provides a substantially pure polypeptide which interacts with the amino acid sequence set forth in SEQ ID NO:12. Also provided is a substantially pure polypeptide which interacts with the amino acid sequence set forth in SEQ ID NO:13. Such amino acid sequence are used as "bait" sequences in yeast two-hybrid screen (See Example 1). Polypeptides identified in such screens are interacting proteins. Interacting proteins can mediate or modulate the activities of intracellular proteins.

A method is provided for identifying a compound that modulates a cellular response mediated by a Glutamate Transporter Associated Protein . The method includes incubating the compound with a cell expressing a Glutamate Transporter Associated Protein and a glutamate transporter protein under conditions sufficient to permit the compound to interact with the cell. The effect of the compound on the cellular response is determined, either directly or indirectly, and a cellular response is then compared with a cellular response of a control cell. A suitable control includes, but is not limited to, a cellular response of a cell not contacted with the compound. The cell may be any cell of interest, including but not limited to neuronal cells, glial cells, cardiac cells, bronchial cells, uterine cells, testicular cells, liver cells, renal cells, intestinal cells, cells from the thymus and spleen, placental cells, endothelial cells, endocrine cells including thyroid, parathyroid, pituitary and the like, smooth muscle cells and skeletal muscle cells. The term "incubating" includes conditions which allow contact between the test compound and the cell of interest. "Contacting" may include in solution or in solid phase.

The cellular response can be an increase in glutamate transport or a decrease in glutamate transport. Glutamate transport can be assessed by measuring glutamate uptake assays (see Example 8) and other assays known in the art.

The cellular response can be an increase in cytoskeletal stability or a decrease in cytoskeletal stability. Cytoskeletal stability can be assessed for example, by examining the formation and maintenance of intracellular protein interaction, cell-surface receptor clustering, clustering of glutamate transporter proteins, and the like. Methods for demonstrating such cellular responses are well known in the art (*e.g.* biochemical methods and histological methods). (See Kornau *et al.* (1997) *Curr. Opin. Neurobiol.* 7:368-373; and Huganir *et al.* (2000) *Trends in Cell Biol.* 10:274-280, each of which are herein incorporated by reference in their entirety and Examples section for additional methodology).

The cellular response can be an increase in chloride flux or a decrease in chloride flux. Chloride flux can be assessed by methods known to those of skill in the art such as electrophysiological methods including, but not limited to, patch clamp analysis.

Glutamate Transporter Associated Proteins contemplated for use in the invention method includes, for example, GTRAP4-41, GTRAP4-48, PCTAIRE-1a, PCTAIRE-1b, and GTRAP3-18. Glutamate transport proteins contemplated for use in the invention method include GLAST, GLT-1, EAAC1, EAAT1, EAAT2, EAAT3, EAAT4 and EAAT5.

In one preferred embodiment of the invention, the glutamate transport protein is EATT4 and the Glutamate Transporter Associated Protein is GTRAP4-41, GTRAP4-48, PCTAIRE-1a or PCTAIRE-1b. In another embodiment of the invention, the glutamate transport protein is EAAC1 and the Glutamate Transporter Associated Protein is GTRAP3-18.

In an embodiment of the invention, the cell expressing a Glutamate Transporter Associated Protein further expresses a RhoGEF protein. The Rho family of GTP-binding proteins regulates the rearrangement of the actin cytoskeleton. At least one Glutamate Transporter Associated Protein has a domain that permits interaction with a a guanine nucleotide exchange factor (GEF).

Compounds which modulate a cellular response include peptides, peptidomimetics, polypeptides, pharmaceuticals, chemical compounds and biological agents, for example. Antibodies, anti-epileptic compounds and combinatorial compound libraries can also be tested using the method of the invention. One class of organic molecules, preferably small organic compounds having a molecular weight of more than 50 and less than about 2,500 Daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups.

The test agent may also be a combinatorial library for screening a plurality of compounds. Compounds such as peptides identified in the method of the invention can be further cloned, sequenced, and the like, either in solution of after binding to a solid support, by any method usually applied to the isolation of a specific DNA

sequence Molecular techniques for DNA analysis (Landegren *et al.*, *Science* 242:229-237, 1988) and cloning have been reviewed (Sambrook *et al.*, Molecular Cloning: a Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Plainview, NY, 1998, herein incorporated by reference).

Candidate compounds are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc., to produce structural analogs. Candidate agents are also found among biomolecules including, but not limited to: peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

A variety of other agents may be included in the screening assay. These include agents like salts, neutral proteins, *e.g.*, albumin, detergents, *etc.* that are used to facilitate optimal protein-protein binding and/or reduce nonspecific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, antimicrobial agents and the like may be used. The mixture of components are added in any order that provides for the requisite binding. Incubations are performed at any suitable temperature, typically between 4 and 40°C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening. Typically between 0.1 and 10 h will be sufficient.

Another embodiment of the invention provides a method for identifying a compound that can inhibit an interaction between a Glutamate Transporter Associated Protein and a glutamate transporter protein. The method includes contacting a Glutamate Transporter Associated Protein with a glutamate transporter protein in the

presence of the compound, and comparing the formation of a Glutamate Transporter Associated Protein-glutamate transporter complex in the presence eof the compound with the formation of the complex in the absence of the compound. Compounds that affect complex formation include peptides, polypeptides, pepidomimetics, chemical compounds and biological agents.

Contacting includes in solution and solid phase. In a preferred embodiment, isolated Glutamate Associated Transporter Proteins are utilized. However, partially purified proteins, fractions of cell extracts, whole cell extracts, or intact cells may be utilized with the method of the invention.

The complex of the Glutamate Associated Transporter Protein and the glutamate transporter protein can be separated from uncomplexed components by conventional means, well known to one of skill in the art. Separation can be accomplished by size separation, physical separation, antibody-mediated separation, or other standard methods. For example, immunoprecipitation or gel electrophoresis can be used to separate Glutamate Transporter Associated Protein-glutamate transporter protein complex from components that are not part of the complex (See Examples section for details).

Also provided is a method of modulating glutamate transport in a subject in need thereof. The method includes administering to the subject a therapeutically effective amount of a compound that modulates expression or activity of a Glutamate Transporter Associated Protein, thereby modulating glutamate transport.

A method is further provided for treating a subject with a disorder associated with glutamate transport comprising administering to the subject a therapeutically effective amount of a compound that modulates Glutamate Transporter Associated Protein activity or interaction with glutamate transporter protein.

Essentially, any disorder that is etiologically linked to glutamate transport or to a Glutamate Transporter Associated Protein could be considered susceptible to treatment with an agent that modulates Glutamate Transporter Associated Protein activity. The disorder may be a neuronal cell disorder. Examples of neuronal cell disorders include but are not limited to epilepsy, neurodegenerative disease (e.g. Alzheimer's disease, Huntington's disease, Amyotrophic lateral sclerosis, Parkinson's disease), spinocerebellar ataxia (SCA), especially of the SCA type 1, multiple

sclerosis, disorders of neurotransmitter metabolism, including GABA metabolism and the like, Alzheimer's disease, Parkinson's disease, stroke, and brain or spinal cord injury/damage, including ischemic injury, and the like. Disorders also include glutamate toxicity, a disorder of memory, a disorder of learning or a disorder of brain development, and the like. Also included are disorders of glutamate-GABA imbalance such as schizophrenia, and the like.

In a preferred embodiment, the Glutamate Transporter Associated Protein is GTAP4-41, GTRAP4-48 or PCTAIRE-1 (including PCTAIRE-1a and PCTAIRE-1b) and the disorder is a disorder of the nervous system such as neurodegeneration or spinocerebellar ataxia type 1.

When the Glutamate Transporter Associated Protein is GTRAP3-18 the disorder is epilepsy or a disorder of GABA metabolism (e.g. tremors, spasticity, schizophrenia), for example.

Treatment can include modulation of Glutamate Transporter Associated Protein expression or activity by administration of a therapeutically effective amount of a compound that modulates Glutamate Transporter Associated Protein or Glutamate Transporter Associated Protein activity. The term "modulate" envisions the suppression of Glutamate Transporter Associated Protein activity or expression when the Glutamate Transporter Associated Protein is overexpressed or has an increased activity as compared to a control. The term "modulate" also includes the augmentation of the expression of Glutamate Transporter Associated Protein when it is underexpressed or has a decreased activity as compared to a control. The term "compound" as used herein describes any molecule, *e.g.*, protein, nucleic acid, or pharmaceutical, with the capability of altering the expression of Glutamate Transporter Associated Protein polynucleotide or activity of Glutamate Transporter Associated Protein. Treatment can inhibit the transcription or translation of a Glutamate Transporter Associated Protein nucleotide sequence, inhibit the interaction of a domain of Glutamate Transporter Associated Protein with its target protein, may increase the avidity of this interaction by means of allosteric effects, may block the binding activity of a domain of Glutamate Transporter Associated Protein or influence other functional properties of Glutamate Transporter Associated Proteins.

Candidate agents include nucleic acids that interfere with expression of Glutamate Transporter Associated Protein , such as an antisense nucleic acid, ribozymes, and the like. Candidate agents also encompass numerous chemical classes wherein the agent modulates Glutamate Transporter Associated Protein expression or activity. For example, when the Glutamate Transporter Associated Protein is GTRAP3-18, the compound can be a polynucleotide having a nucleic acid sequence substantially similar to SEQ ID NO:20 (5'-GAGCGGGGCAAGGTTCAC-3'). A nucleotide encoded by SEQ ID NO:20 is antisense to the nucleic acid sequence of GTRRAP3-18 (See Example 13). GTRAP3-18 can also be modulated by retinoic acid (See Example 14).

When the Glutamate Transporter Associated Protein is GTRAP4-41, GTRAP4-48 or PCTAIRE-1, modulatory compounds include a polynucleotide having a nucleic acid sequence that is substantially similar to an antisense nucleic acid sequence that binds to a polynucleotide encoding GTRAP4-41, GTRAP4-48 or PCTAIRE-1.

Modulation of glutamate transport can be an increase in glutamate transport or a decrease in glutamate transport. When a disorder is associated with an increase in glutamate transport, compounds that decrease glutamate transport can be used. For example, compounds that modulate expression of GTRAP3-18 are contemplated. When a disorder is associated with a decrease in glutamate transport, compound that increase glutamate transport are contemplated. For example, compounds that modulate expression of GTRAP4-41, GTRAP4-48, or PCTAIRE-1 (a and b) are contemplated.

Detection of altered (decreased or increased) levels of Glutamate Transporter Associated Protein expression can be accomplished by hybridization of nucleic acids isolated from a cell of interest with a Glutamate Transporter Associated Protein of the invention. Analysis, such as Northern Blot analysis, are utilized to quantitate expression of Glutamate Transporter Associated Protein , such as to measure Glutamate Transporter Associated Protein transcripts. Other standard nucleic acid detection techniques will be known to those of skill in the art. Detection of altered levels of Glutamate Transporter Associated Protein can also accomplished using assays designed to detect Glutamate Transporter Associated Protein polypeptide. For

example, antibodies or peptides that specifically bind a Glutamate Transporter Associated Protein polypeptide can be utilized. Analyses, such as radioimmune assay or immunohistochemistry, are then used to measure Glutamate Transporter Associated Protein , such as to measure protein concentration qualitatively or quantitatively.

Where a disorder is associated with the increased expression of Glutamate Transporter Associated Protein , nucleic acid sequences that interfere with the expression of Glutamate Transporter Associated Protein can be used. This approach also utilizes, for example, antisense nucleic acid, ribozymes, or triplex agents to block transcription or translation of Glutamate Transporter Associated Protein mRNA, either by masking that mRNA with an antisense nucleic acid or triplex agent, or by cleaving it with a ribozyme in disorders associated with increased Glutamate Transporter Associated Protein. Alternatively, a dominant negative form of Glutamate Transporter Associated Protein polypeptide could be administered.

When Glutamate Transporter Associated Protein is overexpressed, candidate agents include antisense nucleic acid sequences. Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule (Weintraub, 1990, *Scientific American*, 262:40). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA, since the cell will not translate a mRNA that is double-stranded. Antisense oligomers of about 15 nucleotides are preferred, since they are easily synthesized and are less likely to cause problems than larger molecules when introduced into the target cell. The use of antisense methods to inhibit the *in vitro* translation of genes is well known in the art (Marcus-Sakura, 1988, *Anal. Biochem.*, 172:289).

Use of an oligonucleotide to stall transcription is known as the triplex strategy since the oligomer winds around double-helical DNA, forming a three-strand helix. Therefore, these triplex compounds can be designed to recognize a unique site on a chosen gene (Maher, *et al.*, 1991, *Antisense Res. and Dev.*, 1(3):227; Helene, C., 1991, *Anticancer Drug Design*, 6:569).

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences which encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, 1988, *J. Amer. Med. Assn.*, 260:3030). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

There are two basic types of ribozymes namely, *tetrahymena*-type (Hasselhoff, 1988, *Nature*, 334:585) and "hammerhead"-type. *Tetrahymena*-type ribozymes recognize sequences which are four bases in length, while "hammerhead"-type ribozymes recognize base sequences 11-18 bases in length. The longer the recognition sequence, the greater the likelihood that the sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to *tetrahymena*-type ribozymes for inactivating a specific mRNA species and 18-based recognition sequences are preferable to shorter recognition sequences.

When a disorder is associated with the decreased expression of Glutamate Transporter Associated Protein, nucleic acid sequences that encode Glutamate Transporter Associated Protein can be used. An agent which modulates Glutamate Transporter Associated Protein expression includes a polynucleotide encoding a polypeptide of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:22, or a conservative variant thereof. Alternatively, an agent of use with the subject invention includes agents that increase the expression of a polynucleotide encoding Glutamate Transporter Associated Protein or an agent that increases the activity of Glutamate Transporter Associated Protein polypeptide.

In another series of embodiments, the present invention provides transgenic animal models diseases or disorders associated with mutations in the Glutamate Transporter Associated Protein genes. The animal may be essentially any amphibian, reptile, fish, mammal, and the like. Preferably, the transgenic animal is mammalian including rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates. In addition, invertebrate models, including nematodes and insects, may be used for certain applications. The animal models are produced by

standard transgenic methods including microinjection, transfection, or by other forms of transformation of embryonic stem cells, zygotes, gametes, and germ line cells with vectors including genomic or cDNA fragments, minigenes, homologous recombination vectors, viral insertion vectors and the like. Suitable vectors include vaccinia virus, adenovirus, adeno associated virus, retrovirus, liposome transport, neuraltropic viruses, Herpes simplex virus, and the like. The animal models may include transgenic sequences comprising or derived from Glutamate Transporter Associated Proteins including normal and mutant sequences, intronic, exonic and untranslated sequences, and sequences encoding subsets of Glutamate Transporter Associated Protein such as functional domains.

The major types of animal models provided include: (1) Animals in which a normal human Glutamate Transporter Associated Protein gene has been recombinantly introduced into the genome of the animal as an additional gene, under the regulation of either an exogenous or an endogenous promoter element, and as either a minigene or a large genomic fragment; in which a normal human Glutamate Transporter Associated Protein gene has been recombinantly substituted for one or both copies of the animal's homologous Glutamate Transporter Associated Protein gene by homologous recombination or gene targeting; and/or in which one or both copies of one of the animal's homologous Glutamate Transporter Associated Protein genes have been recombinantly "humanized" by the partial substitution of sequences encoding the human homologue by homologous recombination or gene targeting. (2) Animals in which a mutant human Glutamate Transporter Associated Protein gene has been recombinantly introduced into the genome of the animal as an additional gene, under the regulation of either an exogenous or an endogenous promoter element, and as either a minigene or a large genomic fragment; in which a mutant human Glutamate Transporter Associated Protein gene has been recombinantly substituted for one or both copies of the animal's homologous Glutamate Transporter Associated Protein gene by homologous recombination or gene targeting; and/or in which one or both copies of one of the animal's homologous Glutamate Transporter Associated Protein genes have been recombinantly "humanized" by the partial substitution of sequences encoding a mutant human homologue by homologous recombination or gene targeting. (3) Animals in which a mutant version of one of that animal's Glutamate Transporter Associated Protein genes has been recombinantly

introduced into the genome of the animal as an additional gene, under the regulation of either an exogenous or an endogenous promoter element, and as either a minigene or a large genomic fragment; and/or in which a mutant version of one of that animal's Glutamate Transporter Associated Protein genes has been recombinantly substituted for one or both copies of the animal's homologous Glutamate Transporter Associated Protein gene by homologous recombination or gene targeting. (4) "Knock-out" animals in which one or both copies of one of the animal's Glutamate Transporter Associated Protein genes have been partially or completely deleted by homologous recombination or gene targeting, or have been inactivated by the insertion or substitution by homologous recombination or gene targeting of exogenous sequences.

In a preferred embodiment of the invention, there is provided a transgenic non-human animal having a transgene that expresses a Glutamate Transporter Associated Protein-encoding polynucleotide chromosomally integrated into the germ cells of the animal. Animals are referred to as "transgenic" when such animal has had a heterologous DNA sequence, or one or more additional DNA sequences normally endogenous to the animal (collectively referred to herein as "transgenes") chromosomally integrated into the germ cells of the animal. The transgenic animal (including its progeny) will also have the transgene fortuitously integrated into the chromosomes of somatic cells.

Various methods to make the transgenic animals of the subject invention can be employed. Generally speaking, three such methods may be employed. In one such method, an embryo at the pronuclear stage (a "one cell embryo") is harvested from a female and the transgene is microinjected into the embryo, in which case the transgene will be chromosomally integrated into both the germ cells and somatic cells of the resulting mature animal. In another such method, embryonic stem cells are isolated and the transgene incorporated therein by electroporation, plasmid transfection or microinjection, followed by reintroduction of the stem cells into the embryo where they colonize and contribute to the germ line. Methods for microinjection of mammalian species is described in United States Patent No. 4,873,191. In yet another such method, embryonic cells are infected with a retrovirus containing the transgene whereby the germ cells of the embryo have the transgene chromosomally integrated therein. When the animals to be made transgenic

are avian, because avian fertilized ova generally go through cell division for the first twenty hours in the oviduct, microinjection into the pronucleus of the fertilized egg is problematic due to the inaccessibility of the pronucleus. Therefore, of the methods to make transgenic animals described generally above, retrovirus infection is preferred for avian species, for example as described in U.S. Patent No. 5,162,215. If microinjection is to be used with avian species, however, a recently published procedure by Love *et al.*, (Biotechnology, 12, Jan 1994) can be utilized whereby the embryo is obtained from a sacrificed hen approximately two and one-half h after the laying of the previous laid egg, the transgene is microinjected into the cytoplasm of the germinal disc and the embryo is cultured in a host shell until maturity. When the animals to be made transgenic are bovine or porcine, microinjection can be hampered by the opacity of the ova thereby making the nuclei difficult to identify by traditional differential interference-contrast microscopy. To overcome this problem, the ova can first be centrifuged to segregate the pronuclei for better visualization.

The non-human animals of the invention are murine typically (*e.g.*, mouse). The transgenic non-human animals of the invention are produced by introducing "transgenes" into the germline of the non-human animal. Embryonal target cells at various developmental stages can be used to introduce transgenes. Different methods are used depending on the stage of development of the embryonal target cell. The zygote is the best target for microinjection. The use of zygotes as a target for gene transfer has a major advantage in that in most cases the injected DNA will be incorporated into the host gene before the first cleavage (Brinster *et al., Proc. Natl. Acad. Sci. USA* 82:4438-4442, 1985). As a consequence, all cells of the transgenic non-human animal will carry the incorporated transgene. This will in general also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene.

The term "transgenic" is used to describe an animal which includes exogenous genetic material within all of its cells. A "transgenic" animal can be produced by cross-breeding two chimeric animals which include exogenous genetic material within cells used in reproduction. Twenty-five percent of the resulting offspring will be transgenic *i.e.*, animals which include the exogenous genetic material within all of their cells in both alleles. Fifty percent of the resulting animals will include the

exogenous genetic material within one allele and twenty five percent will include no exogenous genetic material.

In the microinjection method useful in the practice of the subject invention, the transgene is digested and purified free from any vector DNA *e.g.* by gel electrophoresis. It is preferred that the transgene include an operatively associated promoter which interacts with cellular proteins involved in transcription, ultimately resulting in constitutive expression. Promoters useful in this regard include those from cytomegalovirus (CMV), Moloney leukemia virus (MLV), and herpes virus, as well as those from the genes encoding metallothionine, skeletal actin, P-enolpyruvate carboxylase (PEPCK), phosphoglycerate (PGK), DHFR, and thymidine kinase. Promoters for viral long terminal repeats (LTRs) such as Rous Sarcoma Virus can also be employed. Constructs useful in plasmid transfection of embryonic stem cells will employ additional regulatory elements well known in the art such as enhancer elements to stimulate transcription, splice acceptors, termination and polyadenylation signals, and ribosome binding sites to permit translation.

Retroviral infection can also be used to introduce transgene into a non-human animal, as described above. The developing non-human embryo can be cultured in vitro to the blastocyst stage. During this time, the blastomeres can be targets for retro viral infection (Jaenich, R., Proc. Natl. Acad. Sci USA 73:1260-1264, 1976). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Hogan, *et al.* (1986) in Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The viral vector system used to introduce the transgene is typically a replication-defective retro virus carrying the transgene (Jahner, *et al., Proc. Natl. Acad. Sci. USA* 82:6927-6931, 1985; Van der Putten, *et al., Proc. Natl. Acad. Sci USA* 82:6148-6152, 1985). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, *supra*; Stewart, *et al., EMBO J.* 6:383-388, 1987). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoel (D. Jahner *et al., Nature* 2-98:623-628, 1982). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of the cells which formed the transgenic nonhuman animal. Further, the founder may contain various retro viral insertions of

the transgene at different positions in the genome which generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germ line, albeit with low efficiency, by intrauterine retroviral infection of the midgestation embryo (D. Jahner *et al., supra*).

A third type of target cell for transgene introduction is the embryonal stem cell (ES). ES cells are obtained from pre-implantation embryos cultured in vitro and fused with embryos (M. J. Evans *et al. Nature* 292:154-156, 1981; M.O. Bradley *et al., Nature* 309: 255-258, 1984; Gossler, *et al., Proc. Natl. Acad. Sci USA* 83: 9065-9069, 1986; and Robertson *et al., Nature* 322:445-448, 1986). Transgenes can be efficiently introduced into the ES cells by DNA transfection or by retro virus-mediated transduction. Such transformed ES cells can thereafter be combined with blastocysts from a nonhuman animal. The ES cells thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal. (For review see Jaenisch, R., *Science* 240: 1468-1474, 1988).

"Transformed" means a cell into which (or into an ancestor of which) has been introduced, by means of recombinant nucleic acid techniques, a heterologous nucleic acid molecule. "Heterologous" refers to a nucleic acid sequence that either originates from another species or is modified from either its original form or the form primarily expressed in the cell.

"Transgene" means any piece of DNA which is inserted by artifice into a cell, and becomes part of the genome of the organism (*i.e.*, either stably integrated or as a stable extrachromosomal element) which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (*i.e.*, foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism. Included within this definition is a transgene created by the providing of an RNA sequence which is transcribed into DNA and then incorporated into the genome. The transgenes of the invention include DNA sequences which encode Glutamate Transporter Associated Protein polypeptide-sense and antisense polynucleotides, which may be expressed in a transgenic non-human animal. The term "transgenic" as used herein additionally includes any organism whose genome has been altered by in vitro manipulation of the early embryo or fertilized egg or by any transgenic technology to induce a specific gene knockout. As used herein, the

term "transgenic" includes any transgenic technology familiar to those in the art which can produce an organism carrying an introduced transgene or one in which an endogenous gene has been rendered non-functional or "knocked out".

Another embodiment of the invention provides a computer readable medium having store thereon a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and sequences substantially identical thereto, or a polypeptide sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:22 and sequences substantially identical thereto.

A further embodiment of the invention provides a computer system comprising a processor and a data storage device wherein said date storage device has stored thereon a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and sequences substantially identical thereto, or a polypeptide sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:22 and sequences substantially identical thereto. The computer system, additionally can contain a sequence comparison algorithm and a data storage device having at least one reference sequence stored on it. The sequence comparison algorithm comprises a computer program which indicates polymorphisms. The term "polymorphism", as used herein, refers to the existence of multiple alleles at a single locus. Polymorphism can be are several types including, for example, those that change DNA sequence but do not change protein sequence, those that change protein sequence without changing function, those that create proteins with a different activity, and those that create proteins that are non-functional.

Embodiments of the invention include systems (*e.g.*, internet based systems), particularly computer systems which store and manipulate the coordinate and sequence information described herein. One example of a computer system 100 is illustrated in block diagram form in Figure 10. As used herein, "a computer system" refers to the hardware components, software components, and data storage components used to analyze the coordinates and sequences as set forth herein. The computer system 100 typically includes a processor for processing, accessing and manipulating the sequence data. The processor 105 can be any well-known type of central processing unit, such as,

for example, the Pentium III from Intel Corporation, or similar processor from Sun, Motorola, Compaq, AMD or International Business Machines.

Typically the computer system 100 is a general purpose system that comprises the processor 105 and one or more internal data storage components 110 for storing data, and one or more data retrieving devices for retrieving the data stored on the data storage components. A skilled artisan can readily appreciate that any one of the currently available computer systems are suitable.

In one particular embodiment, the computer system 100 includes a processor 105 connected to a bus which is connected to a main memory 115 (preferably implemented as RAM) and one or more internal data storage devices 110, such as a hard drive and/or other computer readable media having data recorded thereon. In some embodiments, the computer system 100 further includes one or more data retrieving device 118 for reading the data stored on the internal data storage devices 110.

The data retrieving device 118 may represent, for example, a floppy disk drive, a compact disk drive, a magnetic tape drive, or a modem capable of connection to a remote data storage system (*e.g.*, via the internet) etc. In some embodiments, the internal data storage device 110 is a removable computer readable medium such as a floppy disk, a compact disk, a magnetic tape, etc. containing control logic and/or data recorded thereon. The computer system 100 may advantageously include or be programmed by appropriate software for reading the control logic and/or the data from the data storage component once inserted in the data retrieving device.

The computer system 100 includes a display 120 which is used to display output to a computer user. It should also be noted that the computer system 100 can be linked to other computer systems 125a-c in a network or wide area network to provide centralized access to the computer system 100.

Figure 11 is a flow diagram illustrating one embodiment of a process 200 for comparing a new nucleotide or protein sequence with a database of sequences in order to determine the homology levels between the new sequence and the sequences in the database. The database of sequences can be a private database stored within the computer system 100, or a public database such as GENBANK that is available through the Internet.

The process 200 begins at a start state 201 and then moves to a state 202 wherein the new sequence to be compared is stored to a memory in a computer system 100. As discussed above, the memory could be any type of memory, including RAM or an internal storage device.

The process 200 then moves to a state 204 wherein a database of sequences is opened for analysis and comparison. The process 200 then moves to a state 206 wherein the first sequence stored in the database is read into a memory on the computer. A comparison is then performed at a state 210 to determine if the first sequence is the same as the second sequence. It is important to note that this step is not limited to performing an exact comparison between the new sequence and the first sequence in the database. Well-known methods are known to those of skill in the art for comparing two nucleotide or protein sequences, even if they are not identical. For example, gaps can be introduced into one sequence in order to raise the homology level between the two tested sequences. The parameters that control whether gaps or other features are introduced into a sequence during comparison are normally entered by the user of the computer system.

Once a comparison of the two sequences has been performed at the state 210, a determination is made at a decision state 210 whether the two sequences are the same. Of course, the term "same" is not limited to sequences that are absolutely identical. Sequences that are within the homology parameters entered by the user will be marked as "same" in the process 200.

If a determination is made that the two sequences are the same, the process 200 moves to a state 214 wherein the name of the sequence from the database is displayed to the user. This state notifies the user that the sequence with the displayed name fulfills the homology constraints that were entered. Once the name of the stored sequence is displayed to the user, the process 200 moves to a decision state 218 wherein a determination is made whether more sequences exist in the database. If no more sequences exist in the database, then the process 200 terminates at an end state 220. However, if more sequences do exist in the database, then the process 200 moves to a state 224 wherein a pointer is moved to the next sequence in the database so that it can be compared to the new sequence. In this manner, the new sequence is aligned and compared with every sequence in the database.

It should be noted that if a determination had been made at the decision state 212 that the sequences were not homologous, then the process 200 would move immediately to the decision state 218 in order to determine if any other sequences were available in the database for comparison.

Figure 12 is a flow diagram illustrating one embodiment of a process 250 in a computer for determining whether two sequences are homologous. The process 250 begins at a start state 252 and then moves to a state 254 wherein a first sequence to be compared is stored to a memory. The second sequence to be compared is then stored to a memory at a state 256. The process 250 then moves to a state 260 wherein the first character in the first sequence is read and then to a state 262 wherein the first character of the second sequence is read. It should be understood that if the sequence is a nucleotide sequence, then the character would normally be either A, T, C, G or U. If the sequence is a protein sequence, then it is preferably in the single letter amino acid code so that the first and sequence sequences can be easily compared.

A determination is then made at a decision state 264 whether the two characters are the same. If they are the same, then the process 250 moves to a state 268 wherein the next characters in the first and second sequences are read. A determination is then made whether the next characters are the same. If they are, then the process 250 continues this loop until two characters are not the same. If a determination is made that the next two characters are not the same, the process 250 moves to a decision state 274 to determine whether there are any more characters either sequence to read.

If there are not any more characters to read, then the process 250 moves to a state 276 wherein the level of homology between the first and second sequences is displayed to the user. The level of homology is determined by calculating the proportion of characters between the sequences that were the same out of the total number of sequences in the first sequence. Thus, if every character in a first 100 nucleotide sequence aligned with a every character in a second sequence, the homology level would be 100%.

Homology or identity is often measured using sequence analysis software (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of

Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of homology to various deletions, substitutions and other modifications. The terms "homology" and "identity" in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same when compared and aligned for maximum correspondence over a comparison window or designated region as measured using any number of sequence comparison algorithms or by manual alignment and visual inspection.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequence for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482, 1981, by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443, 1970, by the search for similarity method of person & Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection. Other algorithms for determining homology or identity include, for example, in addition to a BLAST program (Basic Local Alignment Search Tool at the National Center for Biological Information), ALIGN, AMAS (Analysis of Multiply Aligned Sequences), AMPS

(Protein Multiple Sequence Alignment), ASSET (Aligned Segment Statistical Evaluation Tool), BANDS, BESTSCOR, BIOSCAN (Biological Sequence Comparative Analysis Node), BLIMPS (BLocks IMProved Searcher), FASTA, Intervals & Points, BMB, CLUSTAL V, CLUSTAL W, CONSENSUS, LCONSENSUS, WCONSENSUS, Smith-Waterman algorithm, DARWIN, Las Vegas algorithm, FNAT (Forced Nucleotide Alignment Tool), Framealign, Framesearch, DYNAMIC, FILTER, FSAP (Fristensky Sequence Analysis Package), GAP (Global Alignment Program), GENAL, GIBBS, GenQuest, ISSC (Sensitive Sequence Comparison), LALIGN (Local Sequence Alignment), LCP (Local Content Program), MACAW (Multiple Alignment Construction & Analysis Workbench), MAP (Multiple Alignment Program), MBLKP, MBLKN, PIMA (Pattern-Induced Multi-sequence Alignment), SAGA (Sequence Alignment by Genetic Algorithm) and WHAT-IF. Such alignment programs can also be used to screen genome databases to identify polynucleotide sequences having substantially identical sequences. A number of genome databases are available, for example, a substantial portion of the human genome is available as part of the Human Genome Sequencing Project (J. Roach, http://weber.u.Washington.edu/~roach/human_ genome_ progress 2.html) (Gibbs, 1995). At least twenty-one other genomes have already been sequenced, including, for example, *M. genitalium* (Fraser *et al.*, 1995), M. jannaschii (Bult *et al.*, 1996), *H. influenzae* (Fleischmann *et al.*, 1995), *E. coli* (Blattner *et al.*, 1997), and yeast (*S. cerevisiae*) (Mewes *et al.*, 1997), and *D. melanogaster* (Adams *et al.*, 2000). Significant progress has also been made in sequencing the genomes of model organism, such as mouse, *C. elegans*, and *Arabadopsis sp.* Several databases containing genomic information annotated with some functional information are maintained by different organization, and are accessible via the internet, for example, http://wwwtigr.org/tdb; http://www.genetics.wisc.edu; http://genome-www.stanford.edu/~ball; http://hiv-web.lanl.gov; http://www.ncbi.nlm.nih.gov; http://www.ebi.ac.uk; http://Pasteur.fr/other/biology; and http://www.genome.wi.mit.edu.

One example of a useful algorithm is BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, Nuc. Acids Res. 25:3389-3402, 1977, and Altschul *et al.*, J. Mol. Biol. 215:403-410, 1990, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). This algorithm involves first identifying high scoring

sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a word length (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word length of 3, and expectations (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915, 1989) alignments (B) of 50, expectation (E) of 10, M=5, N= -4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, *e.g.*, Karlin & Altschul, Proc. Natl. Acad. Sci. USA 90:5873, 1993). One measure of similarity provided by BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a references sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

In one embodiment, protein and nucleic acid sequence homologies are evaluated using the Basic Local Alignment Search Tool ("BLAST") In particular, five specific BLAST programs are used to perform the following task:

(1) BLASTP and BLAST3 compare an amino acid query sequence against a protein sequence database;

(2) BLASTN compares a nucleotide query sequence against a nucleotide sequence database;

(3) BLASTX compares the six-frame conceptual translation products of a query nucleotide sequence (both strands) against a protein sequence database;

(4) TBLASTN compares a query protein sequence against a nucleotide sequence database translated in all six reading frames (both strands); and

(5) TBLASTX compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

The BLAST programs identify homologous sequences by identifying similar segments, which are referred to herein as "high-scoring segment pairs," between a query amino or nucleic acid sequence and a test sequence which is preferably obtained from a protein or nucleic acid sequence database. High-scoring segment pairs are preferably identified (*i.e.*, aligned) by means of a scoring matrix, many of which are known in the art. Preferably, the scoring matrix used is the BLOSUM62 matrix (Gonnet *et al.*, Science 256:1443-1445, 1992; Henikoff and Henikoff, Proteins 17:49-61, 1993). Less preferably, the PAM or PAM250 matrices may also be used (see, *e.g.*, Schwartz and Dayhoff, eds., 1978, *Matrices for Detecting Distance Relationships: Atlas of Protein Sequence and Structure*, Washington: National Biomedical Research Foundation). BLAST programs are accessible through the U.S. National Library of Medicine, *e.g.*, at www.ncbi.nlm.nih.gov.

The parameters used with the above algorithms may be adapted depending on the sequence length and degree of homology studied. In some embodiments, the parameters may be the default parameters used by the algorithms in the absence of instructions from the user.

Figure 13 is a flow diagram illustrating one embodiment of an identifier process 300 for detecting the presence of a feature in a sequence. The process 300

begins at a start state 302 and then moves to a state 304 wherein a first sequence that is to be checked for features is stored to a memory 115 in the computer system 100. The process 300 then moves to a state 306 wherein a database of sequence features is opened. Such a database would include a list of each feature's attributes along with the name of the feature. For example, a feature name could be "Initiation Codon" and the attribute would be "ATG". Another example would be the feature name "TAATAA Box" and the feature attribute would be "TAATAA". An example of such a database is produced by the University of Wisconsin Genetics Computer Group (www.gcg.com). Alternatively, the features may be structural polypeptide motifs such as alpha helices, beta sheets, or functional polypeptide motifs such as enzymatic active sites, helix-turn-helix motifs or other motifs known to those skilled in the art.

The following examples are intended to illustrate, but not limit, the invention.

EXAMPLE 1

Identification of Proteins Interacting With Glutamate Transporter Proteins

Yeast Two-Hybrid with EAAT4 Yeast two-hybrid screens were performed using the HF7c′ yeast strain harboring the reporter genes HIS 3 and β-galactosidase (β-gal) under the control of GAL4 activation. The final 77 amino acids of EAAT4 (carboxy-intracellular domain SEQ ID NO:12) were subcloned in-frame into pGBT9 (GAL4 binding domain vector, CLONTECH) and used to screen a rat brain cDNA library constructed in pGAD10 (GAL4 activation domain vector, CLONTECH). The plasmids were transformed into HF7c' yeast cells and positive clones selected on triple-minus plates (Leu-, Trp-, His-) and assayed for β-galactosidase activity. Positive clones were co-transformed with either the bait vector or the original pGAD10 vector into yeast cells to confirm the interaction. For a subsequent EAAT4 C-terminal domain analysis, different regions of the final 77 amino acids of EAAT4 were subcloned in-frame into the pGBT9 vector.

Yeast Two-Hybrid Screen with EAAC1 The MATCHMAKER Two-Hybrid System (Clontech) was used for screening. Using the carboxy-terminal intracellular domain of EAAC1 (the carboxy- 87 amino acids, cDNA position 1458-1719; SEQ ID NO:13) as bait in a yeast two-hybrid screen of an adult rat brain cDNA library, 78 clones displaying β-galactosidase activity were identified. Plasmid DNAs were

isolated from positive clones and re-co-transformed with bait cDNA back into yeast to reconfirm the interaction. Restriction and sequencing analyses revealed that ten of these clones with the strongest β-galactosidase activity were identical.

EXAMPLE 2

Isolation and Primary Structure of Glutamate Transporter Associated Proteins

Cloning of full-length GTRAP4-41 and GTRAP4-48 cDNAs. Marathon cDNA amplification (CLONTECH) was used to perform both 5′- and 3′-RACE on cDNA synthesized from rat brain poly(A)$^+$ RNA. The double-stranded cDNA was ligated to the Marathon cDNA Adaptor which contains an adaptor primer (AP1) binding site. The 1.1 kb GTRAP4-41and 1.4 kb GTRAP4-48 cDNA fragments identified using the yeast two-hybrid system were used to design gene-specific primers (GSPs) which could be used in 5′- and 3′-RACE PCR reactions along with the AP1 primer. The RACE products obtained were sequenced and new GSPs designed, generating a series of overlapping RACE products, which were joined together by PCR. Overlapping RACE products were put through ten cycles of denaturation, annealing and extension in the absence of primers. Nested primers were added and the PCR continued for a further 20 cycles to amplify the overlapped template.

GTRAP4-41 AND 4-48 Two independent cDNA clones were isolated and the proteins they encode were named GTRAP4-41 and GTRAP4-48 (for glutamate transporter 4 associated protein). Isolation of the full-length cDNAs by a series of 5' and 3' RACE PCR reactions demonstrated that the largest open reading frame (ORF) for GTRAP4-41 is 7,164 base pairs (SEQ ID NO:1), which encodes a 2,388 amino acid protein (SEQ ID NO:2) with a predicted relative molecular mass (M_r) of 270,958 Da (accession AF225960). A BLAST search of the GenBank database shows that GTRAP4-41 possesses 87% identity with β-spectrin III (accession AB008567). GTRAP4-41 possesses seventeen 16 amino acid spectrin repeats, two α-actinin domains and a pleckstrin homology domain (Fig. 1A).

The largest ORF identified for GTRAP4-48 (accession AF225961) is 4,581 base pairs (SEQ ID NO:3), which encodes a 1,527 amino acid protein (SEQ ID NO:4) with a predicted M_r of 168,698 Da. A BLAST search of the GenBank database shows

that GTRAP4-48 is unique, but it possesses significant homology to the KIAA0380 cDNA-encoded protein (90% identity) and the recently described RhoGEF, p115. GTRAP4-48 posses multiple potential interaction and regulatory domains (Figure 1B). GTRAP4-48 has a PDZ domain, a regulatory G-protein domain, a pleckstrin homology region (PH) and two proline-rich sequences (PRO). These regions have all been implicated in protein-protein interactions by interacting with the C termini of proteins and are thought to be important in the subcellular targeting of the interacting proteins (Katan *et al.*, FEBS Lett. (1999) 452:36-40; LeVine Mol.Neurobiol. (1999); 19:111-149). The function of PH domain is not clearly clarified, several putative functions have been suggested; (1) binding to the β/γ subunit of heterotrimeric G proteins, (2) binding to lipids, (3) binding to phosphorylated Ser/Thr residues, (4) attachment to membrane by an unknown mechanism. The protein multiple PKC phosphorylation sites and one tyrosine kinase phosphorylation site. It contains 3 helix- loop-helix signatures. Finally, the protein contains a separated tandem periodic repeat of SQPEGS of undermined significance.

GTRAP3-18. Following isolation, one clone, E18, was completely sequenced and the protein encoded is named GTRAP3-18 (glutamate transporter EAAC1 associated protein). Clone E18 is a full-length cDNA containing initiation methionine and polyA tail (SEQ ID NO:5). GTRAP3-18 encodes a protein of 188 amino acids (SEQ ID NO:6) with a calculated molecular mass of 22.5 kDa. Protein analysis indicates that it is a very hydrophobic protein with four possible transmembrane domains. Both the carboxy-terminal and amino-terminal domains contain protein kinase C motifs and may be intracellular. JWA protein (Genbank NP006398), a novel human differentially displayed vitamin A responsive gene, is 95% identical to GTRAP3-18, suggesting that GTRAP3-18 is a rat JWA protein homologue.

EXAMPLE 3

Antibodies

Generation of Polyclonal GTRAP4-41 and GTRAP4-48 Antibodies. Affinity purified polyclonal antisera to EAAT4, GTRAP4-41 and GTRAP4-48 were produced using methods identical to previous studies (Rothstein *et al.*, Neuron (1994) 13:713-725. Synthetic peptides corresponding to epitopes of EAAT4 (carboxy-terminal; EKGASRGRGGNESA; SEQ ID NO:14 and amino-terminal;

KNSLFLRESGAGGGCL; SEQ ID NO:15), rat GTRAP4-41 (KRGPAPSPMPQSRSSE; SEQ ID NO:16) and rat GTRAP4-48 (KTPERTSPSHHRQPSD; SEQ ID NO:17) were synthesized. Monospecific antibodies to GTRAP4-41 and 4-48 were produced.

The affinity-purified GTRAP4-41 antibodies recognize a 270 KDa protein in HEK 293T cells transfected with the full-length GTRAP4-41 cDNA and the affinity-purified GTRAP4-48 antibodies recognized a 170 KDa protein in HEK 293T cells transfected with the full-length GTRAP4-48 cDNA

Generation of Polyclonal GTRAP3-18 Antibodies. Affinity purified polyclonal antisera to GTRAP3-18 was produced as described in Rothstein *et al.* (1994) using the amino terminal region epitope, (KFFPGSDRFARPDFR SEQ ID NO:18).

EXAMPLE 4
Expression of Glutamate Transporter Associated Proteins

Fusion proteins and *in vitro* binding. Full-length EAAT4 was subcloned into the *EcoR* I site of the GST-fusion vector pGEX-6P-1 (Pharmacia). Synthesis of recombinant proteins in BL21 cells (Novagen) was induced by 0.1 mM isopropyl β-D-thiogalactoside for 2 hrs at 30°C and purified according to the protocol provided by the manufacturer (Pharmacia). HEK 293T cells were transfected with myc-tagged GTRAP4-41 or GTRAP4-48 and harvested in ice-cold immunoprecipitation (IP) buffer (phosphate buffered saline (pH 7.1), 5 mM EDTA, 1 mM sodium orthovanadate, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.3 μM aprotinin and 1% Triton X-100). The cellular lysate was incubated with GST or GST-EAAT4 immobilized on glutathione-Sepharose-4B, and washed to remove non-specifically bound proteins. Specifically bound proteins were eluted with 2 X SDS loading buffer and analyzed by immunoblotting using an anti-c-myc antibody.

The Glutathione S-transferase (GST) Gene Fusion System (Pharmacia) was used to construct and generate GST-EAAC1 and GST-GTRAP3-18 fusion proteins using pGEX-6P-1 vector as described herein.

GTRAP4-41 and 4-48 Expression Constructs. For transient expression in HEK 293T cells full-length EAAT4 cDNA was subcloned into the *Eco*R I/*Bam*H I

site of the mammalian expression vector pRK5 (Genentech). For co-immunoprecipitation full-length GTRAP4-41 and GTRAP4-48 cDNAs were subcloned into the *Not* I site of a myc-tagged pRK5 vector.

Cell culture and cell transfection. HEK 293T cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in MEM medium supplemented with 10% fetal bovine serum and L-glutamine. For transient transfections cells, were pre-washed with phosphate buffered saline (PBS) and incubated for 10 min at 4°C with 40 mg of each plasmid DNA and 20 mg of salmon sperm DNA. Cells were transfected by electroporation at 300 V and 500 μF with a gene pulser (Bio-Rad) and grown for 48-72 h in either 10 cm culture dishes or plated onto poly-D-lysine coated coverslips in 6-well plates for co-localization studies.

Subcloning, stable transfection and maintenance of cell lines The EAAT4 cDNA was subcloned into pcDNA3.1/Hygro(+) (Invitrogen) using the *EcoR* I restriction site. The plasmid was linearized with *Ssp* I, ethanol precipitated and transfected into HEK 293T cells using the calcium phosphate-DNA precipitation method. 50 mg of DNA per 10 cm dish was used. Cells were incubated with the precipitate in 5% CO_2 at 37°C for 6 hours, the medium containing the precipitate was removed and the cells were washed twice with PBS before adding fresh MEM medium. 48 h after transfection, the cells were split to 50 % confluency and hygromycin (Invitrogen) was added at a concentration of 50 mg/ml. Cell culture medium containing hygromycin was changed every 3 to 4 days. After approximately 2- to 3- weeks of selection, a serial dilution was carried out and cells were plated out, without selection, in a 96-well plate to obtain one cell per well. Several colonies were picked, expanded in selective medium and checked for expression by western blotting. Similarly, the GTRAP4-41 cDNA was cloned into pcDNA3 using the *Not* I restriction site and linearized with *Ssp* I. Selection was with G418 (Mediatech) at a concentration of 5 mg/ml. The GTRAP4-48 cDNA was cloned into the inducible expression vector pIND (Invitrogen) using the *EcoR* I restriction site and linearized with *Sca* I. Selection was with G418 and expression of GTRAP4-48 was induced with 5 mM Ponasterone A (Ecdysone- Inducible Mammalian Expression System, Invitrogen).

Co-immunoprecipitation in heterologous cells. Full-length GTRAP4-41 and GTRAP4-48 cDNAs were subcloned into the *Not* I site of a myc-tagged pRK5 vector and used to transfect the HEK-rEAAT4 cell line by electroporation at 300 V and 500 μF with a gene-pulser (Bio-Rad). After transfection (48-72h), cells were solubilized with 1 ml of ice-cold IP buffer for 2 h at 4°C with rotation and centrifuged to remove cellular debris. 5 μg of rabbit anti-NEAAT4 antibody was added to 0.5 ml of supernatant and incubated overnight at 4°C. 150μl protein A-Sepharose (Pharmacia) was then added for 2 h at 4°C, washed once with IP buffer and three times with IP minus Triton X-100. Bound protein was eluted by boiling in 3 X SDS loading buffer, and analyzed by immunoblotting using the anti-c-myc antibody.

GTRAP3-18 Expression. For transient expression in HEK 293T cells, full-length EAAC1 cDNA was subcloned into the *Eco*R I/*Bam*H I site of the mammalian expression vector pRK5 (Genentech). For co-immunoprecipitation full-length GTRAP3-18 cDNA were subcloned into the *Not* I site of a myc-tagged pRK5 vector.

Cell culture and cell transfection of GTRAP3-18 For transient transfections cells were pre-washed with PBS, incubated for with 40 mg of each plasmid DNA /20 mg of salmon sperm DNA and electroporated as described herein. In some cases, C6 glioma cells, known to naturally express high levels of EAAC1, were transfected with GTRAP3-18.

EXAMPLE 5

To determine the biochemical interaction between Glutamate Transporter Associated Proteins and glutamate transporter proteins, binding and immunoprecipitation assays *in vivo* and *in vitro* were performed.

GTRAP4-41 and 4-48 Immunoprecipitation with Fusion Proteins. Full-length EAAT4 was subcloned into the *EcoR* I site of the GST-fusion vector pGEX-6P-1 (Pharmacia). Synthesis of recombinant proteins in BL21 cells (Novagen) was induced by 0.1 mM isopropyl b-D-thiogalactoside for 2 hrs at 30°C and prepared as a crude bacterial lysate by mild sonication in ice-cold 1 C PBS and solubilization in 1% Triton X-100. Cell debris was removed by centrifugation at 7,000g and the cleared bacterial lysate applied to glutathione-Sepharose-4B (Pharmacia). HEK 293T cells were transfected with myc-tagged GTRAP4-41 or GTRAP4–48 as described herein

and harvested in ice-cold immunoprecipitation (IP) buffer (phosphate buffered saline (pH 7.1), 5 mM EDTA, 1 mM sodium orthovanadate, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.3 mM aprotinin and 1% Triton X-100) with 1% Triton X-100. The cellular lysate was incubated with GST or GST-EAAT4 immobilized on glutathione-Sepharose-4B, and washed to remove non-specifically bound proteins. Specifically bound proteins were eluted with 3 X SDS loading buffer and analyzed by immunoblotting using an anti-c-myc antibody. Bands were visualized by HRP-conjugated secondary antibodies and ECL chemiluminescence (Amersham).

GTRAP4-41 and GTRAP4-48 bind to GST-EAAT4 fusion protein, but do not bind to GST.

Co-immunoprecipitation in heterologous cells Transiently transfected cells (as described herein) were solubilized with 1 ml of ice-cold IP buffer for 2 h at 4°C with rotation and centrifuged to remove cellular debris. 1.2 mg of mouse anti-c-myc antibody was added to 0.5 ml of supernatant and incubated overnight at 4°C. 150 ml protein A-Sepharose (Pharmacia) was then added for 2 h at 4°C, washed once with IP buffer and three times with IP minus Triton X-100. Bound EAAT4 was eluted by boiling in 3 X SDS loading buffer, and analyzed by immunoblotting using the anti-carboxy-terminal EAAT4 antibody.

GTRAP4-41, GTRAP4-48 and KIAA0380 (a close homolog of GTRAP4-48) are coimmunoprecipitated with EAAT4 protein using the amino-terminal anti-EAAT4 antibody.

Immunoprecipitation from cerebellum lysate. Sprague-Dawley (SD) rat cerebellum was dissected, washed with 50 mM Tris-HCl (pH 7.5), 2 mM EDTA and 0.5 mM DTT, and homogenized on ice in buffer containing 20 mM Tris-HCl (pH 7.5), 10% sucrose, 1 mM EDTA, 0.1 mM PMSF, 0.3 mM aprotinin, 1 mM benamidine, 10 mg/ml leupeptine and 10 mg/ml pepstatine. Protein concentration was measured and adjusted to 2-3 mg/ml, and the homogenate was mixed in a 1:1 ratio with the solubilization buffer (homogenization buffer plus 2% Triton X-100). After 2 h, the lysate was spun at 10,000g for 10 min. For each immunoprecipitation, 500 mg of the Triton-lysate was incubated overnight at 4°C with 5 μg of the anti-amino-terminal EAAT4 antibody. Immune complexes were precipitated with protein

A Sepharose (Pharmacia), washed three times with 10 mM Tris-HCl (pH 7.5) and 5 mM EDTA, eluted with 3 X SDS loading buffer, and processed for western blot analysis. The filters were probed with affinity purified rabbit antibodies against GTRAP4-41 and GTRAP4-48.

The biochemical interaction between GTRAP4-41 or GTRAP4-48 and EAAT4 was confirmed using an *in vitro* binding assay. Full-length myc-tagged GTRAP4-41 and GTRAP4-48 were expressed in HEK 293T cells. The solubilized cell extracts were then mixed with bead-linked GST-EAAT4 or GST alone and the bound proteins were eluted. GTRAP4-41 and GTRAP4-48 were specifically retained by the GST-EAAT4 fusion protein, but not by GST alone.

To further assess the interaction between GTRAP4-41 or GTRAP4-48 and EAAT4 in a cellular context, immunoprecipitation studies in transfected heterologous cells were performed. A stable rat EAAT4 expressing cell line was generated in HEK 293T cells (HEK-rEAT4) and transfected with cDNAs encoding myc-tagged GTRAP4-41 and GTRAP4-48. Antibodies directed to the amino-terminus of EAAT4 were used to immunoprecipitate the antigen and any associated protein.

Western blot analysis using an anti-c-myc antibody demonstrates that GTRAP4-41 and GTRAP4-48 coimmunoprecipitate with EAAT4. No coimmunoprecipitation is observed when the precipitating antibody is omitted. Similarly, when the anti-c-myc antibody is used, EAAT4 is co-immunoprecipitated with GTRAP4-41 and GTRAP4-48.

The GTRAP4-41 and GTRAP4-48 interaction with EAAT4 was then studied *in vivo* using solubilized brain preparations. GTRAP4-41 and GTRAP4-48 are co-immunoprecipitated with EAAT4 from brain by antibodies directed at the amino-terminus of EAAT4 but not by antibodies directed at the carboxy-terminus. However, since the site of interaction is within the carboxy-terminus of EAAT4, it is likely that the carboxy-terminal antibodies disrupt the protein-protein interaction. Furthermore, GTRAP4-41 and GTRAP4-48 appear to specifically interact with EAAT4, as GTRAP4-41 and GTRAP4-48 do not co-immunoprecipitate from brain with antibodies directed at the other glutamate transporters, *e.g.,* GLT-1, GLAST and EAAC1. GTRAP4-48 is also not co-immunoprecipitated from brain with antibodies directed at GTRAP4-41. Similarly, GTRAP4-41 is not co-immunoprecipitated with antibodies directed at GTRAP4-48 from HEK 293T cells that were transfected with

full-length myc-tagged GTRAP4-41 and GTRAP4-48, indicating that there is no direct interaction between GTRAP4-41 and GTRAP4-48.

GTRAP3-18 Immunoprecipitation. Coronal sections of rat brain were sliced at 1-2 mm intervals from the cerebellum to the olfactory bulbs. The cortex region was excised from the brain and placed in cold buffer A (50 mM Tris pH 7.5, 2 mM EDTA, 150 mM NaCl, 0.5 mM DTT). The tissue was washed three times in buffer A and the tissue was weighted. The tissue was then homogenized using a blender in 2.5 vol of buffer B (50 mM Tris pH 7.5, 10% glycerol, 5 mM Mg acetate, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF). The particulate material was removed by centrifugation at 15,000 x g for 30 min at 4°C. The supernatant fraction was incubated with Protein A Sepharose beads and primary antibodies as described herein.

The interaction of GTRAP3-18 with EAAC1 was examined using *in vitro* and *in vivo* methods. For in vitro cell-free binding, EAAC1 was expressed as a fusion protein with glutathione S-transferase (GST), and GTRAP3-18 was produced and labeled with [^{35}S]-methionine by in vitro transcription and translation. Purified GST or GST-EAAC1 fusion proteins immobilized on glutathione-Sepharose were incubated with [^{35}S]-labeled GTRAP3-18 protein. GTRAP3-18 specifically binds to immobilized GST-EAAC1 but not to GST alone, indicating an *in vitro* interaction.

Immunoprecipitation experiments were performed to test if EAAC1 and GTRAP3-18 interact *in vivo*. This was first examined in transfected HEK293 cells using amino-terminus c-myc tagged GTRAP3-18. EAAC1 is co-immunoprecipitated with c-myc-GTRAP3-18 in the cell extract prepared from co-expression cells but not from EAAC1 or c-myc-GTRAP3-18 single expression cells. Theses studies with single expression cells show that binding is specific since they rule out the possibility that the results are due to artifact from immunobead nonspecific binding or antibody cross-reaction, respectively. A truncated EAAC1 lacking the interacting carboxy-terminal domain (described herein) is not co-immunoprecipitated with c-myc-GTRAP3-18, further demonstrating the interaction of EAAC1 and GTRAP3-18. This interaction was specific, since EAAT4, another neuronal glutamate transporter subtype, is not immunoprecipitated with c-myc-GTRAP3-18. Identical results are obtained using COS-7 and C6 glioma cell lines.

To study the protein interaction *in vivo*, anti-EAAC1 or GTRAP3-18 polyclonal antibodies were used to immunoprecipitate EAAC1 or GTRAP3-18 from

rat brain extract. Western blotting demonstrates that EAAC1 is specifically co-immunoprecipitated with GTRAP3-18, but not with GLAST, GLT-1 or EAAT4. Similarly, GTRAP3-18 was co-immunoprecipitated with EAAC1. These studies suggest that EAAC1 and GTRAP3-18 interact *in vivo*.

EXAMPLE 6

Identification of the EAAT4- GTRAP4-48 interaction site To evaluate the general/region and or amino acid motif required for the association of GTRAP 4-41 and GTRAP4-48 with EAAT4, a series of two-hybrid screen using different EAAT4, carboxy-terminal truncations and GTRAP proteins as bait was performed. A series of successively larger carboxy terminal deletions of the 77-amino acid carboxy-terminal EAAT4 bait was used to identify regions necessary for ginding of GTRAP4-41 and GTRAP4-48 to EAAT4. Residues 555-561 (QEAELTLP ; SEQ ID NO:9) and 527-534 (GRGGNESVM; SEQ ID NO:10) are required for GTRAP4-41 and GTRAP4-48 binding, respectively (Figure 2).

EXAMPLE 7

Expression and Localization of GTRAP

Expression of GTRAP4-41 and 4-48 protein in brain. GTRAP4-41 and GTRAP4-48 are expressed exclusively in the brain. The highest level of expression of both proteins is in the cerebellum, and somewhat lower levels of expression in the cortex. The apparent molecular weight for GTRAP4-41 is greater than 201 kD; the apparent molecular weight for GTRAP4-48 is less that 200 kD. The native proteins migrate in PAGE identical to proteins expressed in transfected HEK cells

Expression of GTRAP3-18 mRNA in brain Northern analyses of GTRAP3-18 mRNA were performed in brain as well as various body organ tissues. Total RNA was isolated from various rat tissues using a Stratagene RNA isolation kit, separated on 1% agarose gel with 6.7% formaldehyde and blotted onto a nylon membrane (Gene-screen Plus; NEN) by capillary transfer. The blot was hybridized to the full length cDNA probe labeled with ^{32}P by random priming, washed for 2 x 10 min in 2x SSC, 0.1% SDS at 42°C, 1 x 20 min in 0.1x SSC, 0.1%SDS at 65°C and autoradiographed overnight.

GTRAP3-18 mRNA is widely distributed; GTRAP3-18 mRNA is found in the brain, kidney, heart, muscle, liver and cortex. This pattern is consistent with the

distribution of EAAC1 in peripheral tissues (Furuta *et al.*, J Neuroscience (1997) 17:8363-8375; Shayakul *et al.*, Amer.J.Physiol.Renal Physiol. (1997) 42:F1023-F1029). Similarly, GTRAP3-18 protein is expressed in many neural and non-neural tissues, based on immunocytochemical studies using a polyclonal oligopeptide antibody to the amino-terminus of GTRAP3. GTRAP3-18 protein appears to aggregate as multimers. The dimeric form of GTRAP3-18 is the predominant species in tissue homogenates. The dimeric form is also observed when purified GTRAP3-18 protein is detected using the amino-terminal GTRAP3-18 antibody, and when c-myc-GTRAP3-18 protein is detected using anti-c-myc antibodies. Immunohistological analysis of rat brain reveals that GTRAP3-18 protein is expressed widely and is primarily localized to neurons such as cerebellar Purkinje cells which is identical to the distribution of EAAC1. In transfected HEK293 cells, EAAC1 protein appears to be aggregated at the cell membrane, while GTRAP3-18 protein is typically localized to the cell membrane and cytosol, and co-associated with EAAC1 protein at the cell membrane.

Co-localization of GTRAP4-48 or GTRAP4-41 with EATT4 HEK cells, transientlytransfected with EAAT4 (20 μg) and/or GTRAP4-41 (20 μg) and/or GTRAP4-48 (20 μg), were fixed with paraformaldehyde (4%) in phosphate buffer (0.1 M, pH7.4) for 20 min. The cells were then permeabilized with 0.1% Triton X-100, stained with the primary antibodies EAAT4 (1 mg/ml) and c-myc (5 mg/ml) for 1 h, rinsed and incubated with Texas-red and FITC-conjugated secondary antibodies at dilutions of 1:200. Immunofluorescence was viewed with a confocal microscope. Confocal microscopy of transfected cells and of brain sections was performed with a Zeiss LSM 510 laser scanning microscope using fluorescein (Vector, #FI1000) and Texas red (Vector, TI2000) flurochromes.

Both GTRAP4-41 and 4-48 co-localize to membranes domains with EAAT4. GTRAP4-48 expression is associated with a re-distribution of the protein on the membrane in a punctate-like organization.

Immunohistochemistry The cellular localization of GTRAP4-41 and 4-48 was studied in rat brain tissue. Rat brain sections were stained, as previously described Furuta,A.*et al.*, *Neuroscience* **81**, 1031-1042 (1997)) using the following antibodies: carboxy-terminal anti-EAAT4 (1.5 μg/ml), anti-GTRAP4-41 (127 ng/ml)

or anti-GTRAP4-48 (132 ng/ml) antibodies. Texas-red and FITC-conjugated secondary antibodies were used at dilutions of 1:200.

Both GTRAP4-41 and GTRAP4-48 are highly localized to rat cerebellar cortex, with prominent immunolocalization to Purkinje cell somas and dendrites.

GTRAP4-41 and GTRAP4-48 are selectively localized to brain. In rat, GTRAP4-41 and GTRAP4-48 are predominately expressed in the cerebellum, especially the cerebellar cortex with prominent immunolocalization observed in Purkinje cell somas and dendrites, with low level immunoreactivity in striatum, hippocampus and thalamus. Previous studies have shown that EAAT4 is selectively localized to cerebellar Purkinje cells, although low level expression is observed in cerebral cortex, hippocampus and striatum (Furuta *et al., Neuroscience* **81**, 1031-1042 (1997)) Thus, GTRAP4-41, GTRAP4-48 and EAAT4 are co-localized in brain tissue.

GTRAP3-18 mRNA is widely expressed in brain regions and body organs, consistent with the distribution of EAAC1 (Hediger *et al., Am. J Physiol* **277**, F477-F480 (1999); Hediger *Am. J Physiol* **277**, F487-F492 (1999)). Similarly, GTRAP3-18 protein is expressed in many neural and non-neural tissues when protein localization is examined using a polyclonal oligopeptide antibody to the amino-terminus of GTRAP3. GTRAP3-18 protein appears aggregated as multimers. The dimeric form of GTRAP3-18 is the predominant species in tissue homogenates and the dimeric form is also observed when purified GTRAP3-18 protein is detected using the amino-terminal GTRAP3-18 antibody, as well as when c-myc-GTRAP3-18 protein is detected using anti-c-myc antibodies. Immunohistological analysis of rat brain reveals that GTRAP3-18 protein is expressed widely and primarily localized to neurons such as cerebellar Purkinje cells, identical to the expression pattern observed for EAAC1 (He *et al., J Comp Neurol* **418**, 255-269 (2000); Rothstein *et al. Neuron* **13**, 713-725 (1994)).

Localization in heterologous cells In transfected HEK293 cells, EAAC1 protein appears aggregated at the cell membrane while GTRAP3-18 protein is typically localized to the cell membrane and cytosol, and co-associated with EAAC1 protein at the cell membrane.

EXAMPLE 8

GTRAPs Modulate Glutamate Transport

To determine function relationships between GTRAPs and glutamate transporter proteins, sodium-dependent, glutamate transport activity was measured in HEK-rEAAT4 cells transfected with one or more interacting proteins.

To determine the effects of GTRAPs on glutamate transport function, Na+-dependent glutamate transport activity was measured in cells stably transfected with EAAT4, EAAC1, or another glutamate transporter protein and one or more interacter proteins. Stably transfected cells were grown in a monolayer on 6-well plates in MEM supplemented with 10% fetal bovine serum and L-glutamine. Assays were conducted when cells reached ~80% confluency. The wells were pre-rinsed twice with 2 ml of ice-cold tissue buffer (50 mM Tris, 320mM sucrose, pH 7.4). The cells were then incubated for 4 min at 37°C with 1 ml of either sodium- (120 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 2 mM $CaCl_2$, 1 mM KH_2PO_4, 1mM $MgSO_4$, 10% glucose and 10 mM glutamate) or choline- (120 mM choline Cl, 25 mM Tris-HCl pH 7.4, 5 mM KCl, 2mM $CaCl_2$, 1 mM KH_2PO_4, 1mM $MgSO_4$, 10% glucose and 10 mM glutamate) containing buffer labeled with L-[^{3}H]Glu. Uptake was stopped by rinsing three times with 2 ml of ice-cold wash buffer (50 mM Tris pH 7.5 and 160 mM NaCl). Cells were solubilized in 1 ml of 0.1 N NaOH, and 500 ml of lysate was analyzed for radioactivity in a scintillation counter. Na^+-dependent uptake was defined as the difference in radioactivity accumulated in Na^+-containing buffer and in choline-containing buffer. Protein content was measured and glutamate uptake calculated as nmole glutamate per mg of protein. In some cases, homogenates were examined for EAAT4, GTRAP4-41 and GTRAP 4-48 protein.

In an alternative protocol, HEK-rEAAT4 cells transfected with GTRAP4-41 and GTRAP4-48 were grown in a monolayer on 6-well plates in MEM supplemented with 10% fetal bovine serum and L-glutamine. Assays were conducted about 72 h after transfection using the method of Davis (Davis *et al., J. Neurosci.* **18**, 2475-2485 (1998)). GTRAP4-41 and GTRAP4-48 were subcloned into the *Eco RI* site of HSV PrPUC amplicon parent vector (pHSVPrPUC) (Geller,A.I., *et al., Proc Natl Acad Sci U. S. A* **87**, 8950-8954 (1990)). 3.6 μg of amplicon vector DNA and 25 μg pBAC-V2 DNA were used to transfect 2-10^7 baby hamster kidney cells according to previously published methods (Stavropoulos and Strathdee, *J. Virol.* **72**, 7137-7143 (1998)).

Virus was harvested about 72 hrs after transfection and titered as previously described (Bowers *et al. Mol. Ther.* **1**, 294-299 (2000)). 2 x 10^5 expression particles were injected intra-cisternally into male Sprague-Dawley rats (250 g) obtained from Zivic Miller. About 48 h after injection, the rats were sacrificed and synaptosomal preparations of the cerebelli were prepared using a polytron. Glutamate transport was measured by methods described herein.

GTRAP4-41 and GTRAP4-48 produce a two- to four-fold increase in glutamate transport, respectively. The co-expression of GTRAP4-41 and GTRAP4-48 results in a further increase in glutamate uptake. Kinetic analysis indicates that GTRAP4-41 and GTRAP4-48 produced an increase in the V_{max} of glutamate transport activity (Fig. 3). There is also a small increase in the K_m values when GTRAP4-41 and GTRAP4-48 are co-expressed, but these are not statistically significant, suggesting that the interacting proteins do not alter the affinity of the transporter for glutamate. GTRAP4-41 and GTRAP4-48 may therefore enhance glutamate transport either via an increase in the catalytic rate of the transporter or via an increase in cell surface availability. Results are presented in Figure 3. GTRAP41 and GTRAP48 expression in HEK-rEAAT4 cells increase glutamate uptake significantly over vector alone (VA) transfected cells. Data in Fig. 3A are the mean ± SEM of at least four independent observations and were compared by students *t* test, (** $p < 0.005$). Concentration dependence of Na^+-dependent L-[^{3}H]-glutamate uptake was assayed in the presence of increasing concentrations of glutamate. In Fig. 3B, the values are expressed as the mean ± SEM of a representative experiment carried out in triplicate. Kinetic data shows that GTRAP41 (▯ increases the V_{max} from 222 to 605 pmol/mg/min and increases the K_m slightly from 7 to 11 μM, compared to EAAT4 alone (▲). GTRAP48 increases the V_{max} from 208 to 512 pmol/mg/min (•) and increased the K_m from 10 to 13 μM.

To test if GTRAP3-18 modulates EAAC1 function, sodium-dependent [^{3}H]-glutamate transport was studied in HEK293 cells co-expressing both proteins, 72 hrs after transfection (Rothstein *et al. Neuron* **16**, 675-686 (1996); Lin *et al., Neuron* **20**, 589-602 (1998)). Total glutamate transport progressively decreases with increasing GTRAP3-18 protein expression (Fig. 4). GTRAP3-18 negatively modulates EAAC1-mediated glutamate transport. Glutamate transport was studied in HEK293 cells transfected with plasmids indicated in Fig . 4 . GTRAP3-18 inhibited EAAC1-

mediated transport, but had no effect on EAAT4 (n=6). The co-expression of GTRAP-3-18 has no effect on total EAAC1 protein expression. Analysis of HEK293 cells by confocal microscopy and surface biotinylation reveal no alteration in the membranous localization of EAAC1. Superoxide dismutase (SOD1) was used as a control. Eadie-Scatchard plot of glutamate transport in transfected HEK293 cells reveals a 4-10 fold decrease in affinity (n=4). This effect is specific for EAAC1; co-expression of GTRAP3-18 with EAAT4 has no effect on transport activity. The inhibition of transport is not due to a decrease of EAAC1 protein level by the co-expression of GTRAP3-18, as quantitated by Western blotting. Similarly, the loss of EAAC1 activity is not due to altered protein trafficking; even at high levels of GTRAP3-18 expression, when little EAAC1-mediated transport is observed, EAAC1 surface expression is unaltered as determined by surface biotinylation and confocal microscopy.

To evaluate the biochemical nature of altered transport, kinetic analyses were performed with HEK293 cells co-expressing EAAC1 and GTRAP3-18. EAAC1 and GTRAP3-18 co-expressing cells show a decrease in affinity (K_m =40 μM, Vmax= 0.99 nmol/min/mg protein; n=4, P<0.01) without a shift in the V_{max} when compared to cells only expressing EAAC1 (K_m =9 μM; V_{max}=1.02 nmol/min/mg protein; Figure 4A). Similar results are observed with other cell lines (COS7 and C6 glioma).

EXAMPLE 9

Cell Surface Levels of GTRAPs and Cytoskeletal Stability

To examine changes in the cell surface levels of EAAT4, a cell membrane-impermeant biotinylation reagent to label cell surface proteins selectively. Biotinylation of cell surface proteins was performed as described in Duan et al. (Duan, *et al., J. Neurosci.* **19**, 10193-10200 (1999)). SOD1 was used to control for total protein and to determine whether the biotinylation reagent labels proteins in the intracellular compartment. Densitometry was performed using the NIH Image program.

The total amount of EAAT4 is increased when GTRAP4-41 and GTRAP4-48 are co-expressed (Fig. 5). In contrast the total amount of SOD1, a control for total amount of protein loaded, is unaltered or decreased in the GTRAP4-41 and GTRAP4-48 samples, respectively. The majority of the EAAT4 is biotinylated, indicating that it is at the cell surface. However the percentage of total EAAT4 that is at the cell

surface remains the same when GTRAP4-41 and GTRAP4-48 are co-expressed. Taken together, these results indicate that GTRAP4-41 and GTRAP4-48 stabilize/anchor EAAT4 at the cell membrane, making it less likely to be internalized and subsequently degraded, rather than causing an increased trafficking of EAAT4 to the cell surface.

However it is also possible that there is increased expression of the cell's native gene. To address this question cells were treated 48 hrs after transfection with cycloheximide, an inhibitor of protein synthesis. Quantification by densitometry shows that 12 hrs after treatment, the EAAT4 protein in HEK-rEAAT4 cells is reduced to 54 ± 0.6% of the level prior to cycloheximide treatment. In contrast, 81 ± 2% and 74 ± 1.7% of the EAAT4 protein remains after 12 hrs when GTRAP4-41 and GTRAP4-48 are coexpressed, respectively. These results provide evidence that GTRAP4-41 and GTRAP4-48 do stabilize EAAT4 at the membrane.

EXAMPLE 10

GTRAP- Glutamate Transport Protein Interactions

To determine whether the EAAT4/GTRAP4-48 interaction is required to mediate the increase in EAAT4 activity, HEK-rEAAT4 cells were transfected with GTRAP4-48 constructs lacking the last 155 amino acids which were pulled out by EAAT4 in the yeast two-hybrid screen . The carboxy-terminally truncated GTRAP4-48 had only a modest effect on stimulating EAAT4 activity, indicating that the protein-protein interaction is responsible for the majority of the increase in uptake activity. HEK-rEAAT4 cells were co-transfected with GTRAP4-48 and a myc-tagged cDNA construct encoding the last 77 amino acids of EAAT4 to disrupt the interaction of GTRAP4-48 with full-length EAAT4. Co-expression of this construct inhibits the GTRAP4-48 mediated effect by approximately 25%, but co-expression of a smaller construct (residues 1452 to 1578), which lacked the GTRAP4-48 binding domain, has no effect. Taken together these results indicate that the EAAT4/GTRAP4-48 interaction plays an important role in the modulation of EAAT4 uptake activity.

These results are summarized in Figure 6. Fig 6A shows results of experiments in which HEK-rEAAT4 cells were transfected with a GTRAP48 construct that lacked the C-terminus (domain that interacts with EAAT4). Disruption of the EAAT4/GTRAP48 interaction significantly reduces the GTRAP48-mediated increase in EAAT4 uptake activity (* $p< 0.05$). Disruption of the protein-protein

interaction by overexpression of the EAAT4 C-terminus in HEK-rEAAT4 cells transfected with GTRAP48. The GTRAP48-mediated effect on EAAT4 activity was reduced by ~25% (** $p< 0.005$; Fig 6B). Na^+-dependent L-[^{3}H]-glutamate was assayed in triplicate and values are expressed as the mean ± SEM of six independent experiments. GTRAP41 and GTRAP48 significantly increased glutamate uptake *in vivo* (* $p < 0.05$; Fig 6C) .

The physiological relevance of GTRAP4-41 and GTRAP4-48 on EAAT4 uptake activity *in vivo* was subsequently examined by the intra-cisternal injection of HSV amplicon vectors expressing GTRAP4-41 and GTRAP4-48. Cerebellar glutamate uptake was measured 48 hrs after injection and found to be elevated when GTRAP4-41 and GTRAP4-48 are expressed but not when the control HSVlac amplicon vector was injected (Fig. 6C). Dihydrokainic acid (DHK), an inhibitor of GLT-1 mediated glutamate transport, has no effect on cerebellar glutamate uptake, ruling out any involvement of GLT-1. Unfortunately there is no method to distinguish functionally between GLAST, EAAC1 and EAAT4 . However it has been have shown that GTRAP4-41 and GTRAP4-48 do not interact directly with any other transporter, it is likely that the observed increase in uptake is attributed to an increase in EAAT4 activity. Western blot analysis confirms increased expression of GTRAP4-41 and GTRAP4-48 in the cerebellum following the injections.

EXAMPLE 11

Clustering of Glutamate Transporter Proteins at Synapses

To examine whether GTRAPs are involved in, or associated with, the clustering of EAAT4 at synapses primary cultures of rat Purkinje cell neurons were examined immunocytochemically. Rat brain sections were stained, as previously described (Furuta *et al.,* Neurosciences 81:1031-1042 (1997)) using the following antibodies: carboxy-terminal anti-EAAT4 (1.5 μg/ml), anti-GTRAP4-41 (127 ng/ml) or anti-GTRAP4-48 (132 ng/ml) antibodies. Texas-red and FITC-conjugated secondary antibodies were used at dilutions of 1:200.

EAAT4 and GTRAP4-41 immunoreactivity is observed throughout the soma and dendrites but is also found to colocalize in distinct clusters. Labeling with synaptophysin, a presynaptic protein, reveals that 71% of synapses possessed clusters of EAAT4 and GTRAP4-41 [n=12]. This perisynaptic distribution of GTRAP4-41 correlates with earlier EM studies that showed that EAAT4 is a perisynaptic protein.

Similar studies could not be carried out for GTRAP4-48 due to low level of expression at this early developmental stage.

EXAMPLE 12

Interaction with Rho: Since GTRAP4-48 possesses areas of homology to p115 and PDZRhoGEF, two novel RhoGEFs that selectively activate Rho (Hart *et al., J. Biol. Chem.* **271**, 25452-25458 (1996), Fukuhara *et al., J. Biol. Chem.* **274**, 5868-5879 (1999)), interaction of GTRAP4-48 with the Rho family of GTPases was investigated.

Guanine nucleotide exchange assay. Small G proteins GST-RhoA, GST-CDC42 and GST-Rac were expressed in bacterial cells and affinity purified to ~80% purity using a glutathione column. Twenty pmoles of each protein were incubated with 100 pmoles GTPγS for 10 min at 30°C with varying concentrations of full-length GTRAP4-48 or p115. The binding reactions were filtered through BA-85 nitrocellulose and the amount of GTPγS bound to small G protein was quantitated by scintillation counting of the dried filters. The amount of GTPγS that bound to GST-RhoA, GST-Cdc42 and GST-Rac in the presence of full-length GTRAP4-48 or p115 was measured.

GTRAP4-48, like p115, demonstrates a specific guanine nucleotide exchange activity for Rho (Fig. 7). Co-immunoprecipitation assays also show that GTRAP4-48 interacts with the active form (in the presence of aluminium fluoride) of the $G\alpha_{13}$ subunit and therefore, may act as a link between G-protein coupled receptors and their downstream targets. However, unlike p115, regulation of the GTRAP4-48 RhoGEF activity by $G\alpha_{13}$ nor the stimulation of the GTPase activity of $G\alpha_{13}$ by GTRAP4-48 could be demonstrated. Rho is known to regulate the remodeling of the actin cytoskeleton through various actin-binding proteins, although the mechanism is not yet well characterized (Hall, *Science* **279**, 509-514 (1998)).

Since GTRAP4-48 can activate Rho, expression of GTRAP4-48 was studied to determine if it could induce the reorganization of the actin cytoskeleton and whether it alters the distribution of GTRAP4-41, a possible actin binding protein. When GTRAP4-41 is expressed alone there is a close relationship between actin and GTRAP4-41 at the cell membrane but there are very few organized actin filaments. Conversely, when GTRAP4-41 and GTRAP4-48 are co-expressed, GTRAP4-41 is

found to co-localize with actin in structures that resembled actin-stress fibers, a typical Rho-dependent effect. Overexpression of GTRAP4-48 also induces the formation of membrane ruffling and filopodia, suggesting some degree of cross-talk between the small GTPases, as these are typical Rac and Cdc42 dependent effects. These results indicate that there is a close relationship between GTRAP4-48 and the reorganization of GTRAP4-41 and the actin cytoskeleton.

EXAMPLE 13

Antisense Treatment with GTRAP3-18

To demonstrate tonic modulation of EAAC1 activity by GTRAP3-18, antisense oligomers were used to lower GTRAP3-18 expression in HEK293 cells. Western blot analyses and glutamate uptake assays reveal endogenous expression of EAAC1 and GTRAP3-18 protein in HEK cells, but no expression of other transporter subtypes, *e.g.*, GLAST, GLT-1, or EAAT4. Antisense oligomers, targeted to the 5'-GTRAP3-18 transcript, were transfected into HEK293 cells.

Antisense oligomers specifically reduced endogenous GTRAP3-18 protein level (Fig. 8A, gray bars); EAAC1 protein level was not affected. Significantly, glutamate transport activity was concomitantly elevated with the reduction of GTRAP3-18 protein level (black bars).

To examine modulation of EAAC1 by GTRAP3-18 *in vivo*, GTRAP3-18 antisense oligomers were administered intraventricularly. Sequences for the novel phosphodiester oligonucleotides used were: sense GTRAP3-18: 5'-GTGAACCTTGCCCGCTC-3', antisense GTRAP3-18: 5'-GAGCGGGGCAAGGTTCAC-3' Oligonucleotides (5μg/μL), separately or in combination were administered intraventricularly over 3-11 days, by mini-osmotic pumps (Alza Corp., Palo Alto, CA) as described previously (Rothstein *et al.*, (1994)).

Eleven days of antisense treatment resulted in a reduction of GTRAP3-18 protein level and a significant increase in cortical glutamate uptake, whereas glutamate uptake was not altered in sense oligomer-treated animals (Figure 8B). The effect was due to increased EAAC1-mediated transport because it was not altered by dihydrokainic acid (DHK), an inhibitor of GLT-1-mediated glutamate transport (Robinson *et al. (1998)* Neurochem. Int. 33:479-491). In kinetic studies of DHK-insensitive, cortical glutamate uptake from antisense-treated animals, the apparent

affinity for glutamate was increased (antisense K_m=10μM, V_{max}=1.08 nmol/min/mg protein) compared to artifical CSF or sense treated control animals (control K_m=19.7 μM; V_{max}= 1.08 nmol/min/mg protein; Figure 8C). These results suggest that GTRAP3-18 negatively modulates EAAC1 glutamate transport activity in vivo.

EXAMPLE 14

Regulation of GTRAP3-18 by Retinoic Acid

Human GTRAP3-18 (JWA protein) was originally identified as a retinoic acid responsive gene. Therefore, retinoic acid was tested for its ability to up-regulate GTRAP3-18 expression and consequently inhibit EAAC1-mediated glutamate transport in HEK293 cells. Retinoic acid induces a large increase in GTRAP3-18 expression, over a non-toxic dose range from 1-10 μM. A significant decrease in glutamate transport activity paralleled the increase of GTRAP3-18 protein level (Figure 9). The loss of transport activity is not due to changes in EAAC1 protein level (Figure 9A) or the cellular membrane localization of EAAC1 protein by retinoic acid as examined by fluorescent microscopy. To confirm that loss of transport activity was specifically due to GTRAP3-18 and not by other factors induced by retinoic acid or direct effects on EAAC1, a truncated EAAC1 cDNA, lacking the last 93 amino acids, was constructed. The truncation corresponded to the region used as bait in yeast two-hybrid screening, and was not able to interact with GTRAP3-18. Nevertheless, after transient expression in HEK293 cells, the truncated EAAC1 transported glutamate. Importantly, retinoic acid treatment does not alter activity of the truncated EAAC1 protein; even though GTRAP3-18 protein expression was markedly elevated (Figure10B). Thus, the loss of transport activity by retinoic acid was the result of GTRAP3-18 induction. Interestingly, truncated EAAC1 has increased glutamate transport activity compared to wild-type. Truncated EAAC1 had a K_m of 5.4 μM, which was greater than a three-fold increase in affinity compared to wild-type EAAC1 (Km=17 μM; Figure 9C). This could reflect lack of natural inhibition of the truncated protein EAAC1 by endogenous GTRAP3-18 – results similar to the effects of GTRAP3-18 antisense treatment (Figure 9E).

To test this hypothesis in vivo, retinoic acid was infused intraventricularly (1-20μM; 0-20 pmol/μL). After 4 days of treatment, cortical GTRAP3-18 protein expression was increased in a dose dependent manner, and this was associated with a

significant decrease of total glutamate uptake (Figure 9D, top panel). This effect is specifically due to decreased EAAC1-mediated transport because it was not altered by the glutamate transport inhibitor dihydrokainic acid, at a concentration that predominantly effects GLT-1 (Robinson *et al.* (1998)). Kinetic analysis of DHK-insensitive, cortical glutamate transport from animals treated four days with intraventricular retinoic acid reveals a 4-fold decrease in affinity compared to control transport (Figure 9E) which is very similar to that seen in vitro (Figure 9B). In addition, retinoic acid inhibition of glutamate transport could be reversed in vivo; chronic intraventricular treatment with antisense GTRAP3-18 oligomer (50-100 ng/day, for 7-10 days) blocks the retinoic acid (2.5 μM) induction of GTRAP3-18, and also blocks the inhibition of glutamate transport seen with retinoic acid treatment (Figure 9D, top panel). Retinoic acid had no effect on glutamate transport by cells expressing GLT-1 or EAAT4.

EXAMPLE 15

Glutamate Transporter Associated Protein PCTAIRE-1

The glutamate transporter EAAT4 possesses high affinity Na^+-dependent transport activity, as well as a unique ligand-gated Cl^- conductance. Largely located in the somatodendritic compartment of the cerebellar Purkinje cell, altered function of EAAT4 may contribute to the pathogenesis of spinocerebellar ataxia and alcoholic cerebellar degeneration. In an effort to delineate possible regulatory mechanisms of EAAT4, we have identified glutamate transporter associated proteins (GTRAPs). Using the amino terminus of rat EAAT4 as bait in a yeast two-hybrid screen, an interacting protein was isolated. Subsequent sequence analysis identified the GTRAP as PCTAIRE-1, a serine/threonine kinase related to the cyclin-dependent kinase 2 (cdk2) family. In vitro and in vivo co-immunoprecipitations from rat cerebelli were performed, confirming specificity of interaction; co-localization of EAAT4 and PCTAIRE-1 within the cerebellum was determined using immunofluorescence. In order to investigate regulatory physiology of the PCTAIRE-1/EAAT4 interaction, co-transfection experiments and pharmacologic manipulation were carried out. PCTAIRE-1, although a member of the cdk2 family, is present mainly in terminally differentiated tissues such as brain. It has been shown to interact with members of signal transduction cascades (14-3-3 proteins) and components of cellular protein networks such as p11, a target for annexin II. These data suggest a mechanism by

which EAAT4 may be linked to cellular regulatory machinery via the GTRAP PCTAIRE-1.

Methods. Yeast Two-Hybrid Screening: Screening was performed using the AH109 yeast strain harboring the reporter genes ADE/HIS, as well α- and β-galactosidase. The initial 60 amino acids of EAAT4 were subcloned in-frame into pGBKT7 (GAL4 binding domain, CLONTECH), and used to screen a rat brain cDNA library constructed in pACT2 (GAL4 activation domain, CLONTECH). Following cotransformation and verification of true positive colonies, DNA sequence analysis was performed. Obtained sequences were compared to known GENBANK submissions, resulting in identification of a true postive with with >95% homology to the final 201 amino acids of rat PCTAIRE-1.

Creation of expression constructs: Full length rat PCTAIRE-1 was isolated from a rat brain cDNA library via PCR amplification using upstream and downstream primers based on the known PCTAIRE sequence. Products were cloned into pCMVmyc tagged eukaryotic expression vector (CLONTECH), and expression verified by western blotting.

SUB A3

Two PCTAIRE-1 proteins are identified. PCTAIRE-1a is encoded by PCTAIRE-1 nucleic acid sequence, nucleotides 251-452 and 584-1872 (SEQ ID NO:8) and PCTAIRE-1b is encoded by PCTAIRE-1 nucleic acid sequence, nucleotides 487-1872 (SEQ ID NO:22).

Immunoprecipitations. In-vitro coimmunoprecipitaions were performed on stably transfected HEK cells expressing EAAT4. Vector DNA or myc-tagged PCTAIRE vector was then introduced. Following expression, cells were solubilized with ice-cold IP buffer and centrifuged to remove cellular debris. 0.5 ml of supernatant was then incubated with or without 1.5 μg anti-c-myc antibody (Boehringer-Mannheim). Complexes were then isolated using protein-A Sepharose, washed four times with IP buffer with and without Triton X-100, and visualized using SDS-PAGE. EAAT4 western blotting was performed using affinity purified rabbit polyclonal Ab at 1:200 dilution.

In-vitro coimmunoprecipitation of EAAT4 with myc tagged antibody in EAAT4 expressing HEK cells transfected with myc-labeled PCTAIREs is demonstrated.

In-vivo coimmunoprecipitation was performed using the cerebellum of a 5 day-old Sprague-Dawley rat. Homogenization was performed on ice using a buffer containing 20 MM Tris-HCl (pH 7.5), 10% sucrose, 1 mM EDTA, and protease inhibitors. The homogenate was mixed 1:1 with buffer containing 2% Triton X-100, and solubilized for 2 h at 4°C. 0.5 mg of protein was used for each immunoprecipitation. Antibodies to the carboxy terminal EAAT4 (2.0 μg), as well as antibody to the transporter GLT (2.0 μg) were used. In addition, blocking peptide was presorbed to EAAT4 Ab to further demonstrate specificity. Western blotting was performed using PCTAIRE-1 antibody at 1:200 dilution (Santa Cruz).

In-vivo coimmunoprecipitation of PCTAIRE by EAAT4 is found in neonatal rat cerebellum. A PCTAIRE doublet (62 and 68 kDa) is recovered by immunoprecipitation with c-terminal EAAT4 Ab, and inhibited by preabsorption of EAAT4 Ab with blocking peptide.

Transfection of EAAT4 expressing HEK cells with PCTAIRE results in diminished Na^+-dependent glutamate uptake. HEK cells and EAAT4 expressing HEK cells were transfected with 1.0 μg of pCMV PCTAIRE plasmid, and allowed to express for 48 hours. Cell monolayers were then washed with tissue buffer (50 mM Tris, 320 mM sucrose, pH 7.4). The cells were then incubated for 4 min at 37°C with 1 mL of either sodium-(120 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 2 mM $CaCl_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 10% glucose and 10 μM glutamate or choline-(120 mM choline-Cl, 25 mM Tris-HCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 10% glucose and 10 μM glutamate) containing buffer. Glutamate uptake assays were then performed using L-[^{3}H]-Glutamate in the presence and absence of Na^+. After rinsing, cells were lysed in 0.1 N NaOH and lysate radioactivity measured using a scintillation counter. Protein content was measured and glutamate uptake calculated as the difference between Na^+ containing and sodium free values per mg of protein.

Inhibition of Na^+-dependent glutamate uptake by PCTAIRE is reversible using the cdk2 inhibitor olomucine. HEK cells expressing EAAT4 were transfected with 1.0 μg of pCMV PCTAIRE plasmid as described above, and allowed to express for 48 hours. Prior to glutamate uptake assay, cells were treated with 100 μM olomucine for 30 minutes at 37°C as indicated. Olomocine belongs to a class of cyclin

dependent kinase inhibitors which inhibit activity via competition at the ATP binding site.

Immunofluorescence microscopy displays colocalization of EAAT4 and PCTAIRE in the Purkinje cell layer of the rat cerebellum. A five day-old rat pup was perfusion fixed, the brain extracted, and 25 μm sections stained with antibodies to c-terminal EAAT4 (1.5μg/mL) and PCTAIRE-1 (1.5μg/mL). Prominent double-labeling is evident in the Purkinje cell layer, especially the cell soma, where EAAT4 is known to be present during the early postnatal period.

These results indicate that the serine/threonine kinase PCTAIRE interacts with the amino-terminus of the glutamate transporter EAAT4. This interaction results in downregulation of Na^+-dependent glutamate uptake, and this process is reversible using an inhibitor of cyclin dependent kinases. In addition, immunofluorescence reveals that both EAAT4 and PCTAIRE localize to the cerebellum, particularly the purkinje cell layer. Although PCTAIRE bears homology to the family of cyclin dependent kinases involved in proliferation, it is found mainly in terminally differentiated tissues such as brain. Other EAAT4 interacting proteins have recently been identified, both of which interact at the carboxy-terminus, and upregulate glutamate uptake. GTRAP 41 is a new member of the β-III spectrin family, and is likely an actin-binding protein. GTRAP4-48 is a novel RhoGEF that may provide a link between the heterotrimeric G-proteins and small GTP-binding proteins of the Rho family. Together with PCTAIRE, these interactors may regulate glutamate uptake through EAAT4.

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.